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We have expanded our previous studies to catalog the clinical data, distribution of PCa involvement, morphology, immunophenotypes, and gene expression from 30 rapid autopsies of men who died of hormone refractory PCa. A Tissue microarray (TMA) and cDNA microarray was constructed and quantitatively evaluated for expression of several biomarkers and genes. Comparisons were made between patients as well as within the same patient. No consistent differences were found between bone and soft tissue sites that could explain the predilection of prostate cancer cells to metastasize to bone. This suggests that biologic differences leading to the predominant pattern of prostate cancer metastasis to bone may be related to the bone "soil" rather than the prostate cancer "seed". Metastatic hormone refractory PCa has a heterogeneous morphology, immunophenotype, and genotype, demonstrating that "metastatic disease" is a group of diseases even within the same patient. An appreciation of this heterogeneity is critical to evaluating diagnostic and prognostic biomarkers as well as designing therapeutic targets for advanced disease. Further investigation of the bone versus soft tissue stroma in cancer versus normal tissue is warranted.

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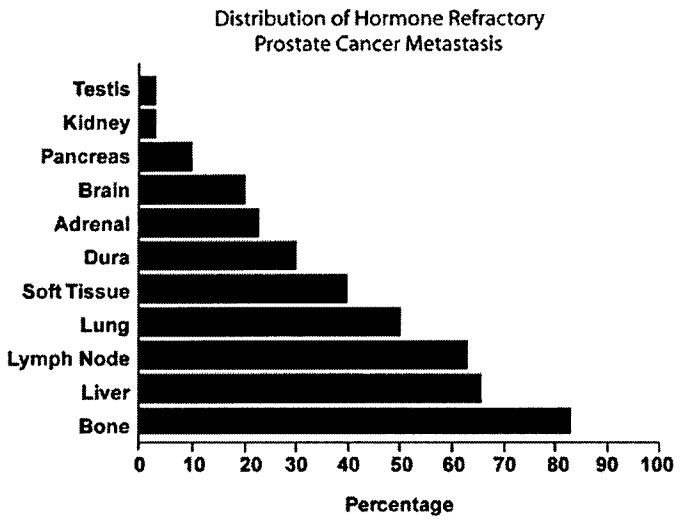
Introduction:

In the United States, prostate cancer (PCa) remains the most common solid tumor malignancy in men, causing approximately 30,000 deaths in 2004 [1]. With the goal of trying to better understand the biology of prostate tumorigenesis and metastasis, we have developed a rapid autopsy program to collect tumors from multiple sites including solid organs and bone in order to perform molecular studies to better delineate PCa progression [2]. Our group has used these samples with a combination of cDNA expression and tissue microarray analysis approaches to identify novel PCa biomarkers, including EZH2, MTA1, PIM1, and AMACR using a supervised analysis comparing androgen independent to localized PCa samples [3-7]. As part of these studies we have begun to investigate the biology underlying the predisposition of prostate cancer to grow preferentially in osseous sites. We have isolated cell lines from bone and soft tissue sites, VCaP and DuCaP, and utilized these to investigate whether the gene expression of these cell lines gave us clues as to whether the prostate cancer cell "seed" demonstrated characteristics that would explain metastasis to bone versus soft tissue [8-11]. We also investigated the characteristics of a small number of bone versus soft tissue metastases and found preliminary differences in their gene expression patterns. Our original statement of work and our subsequent interim reports have detailed our investigations.

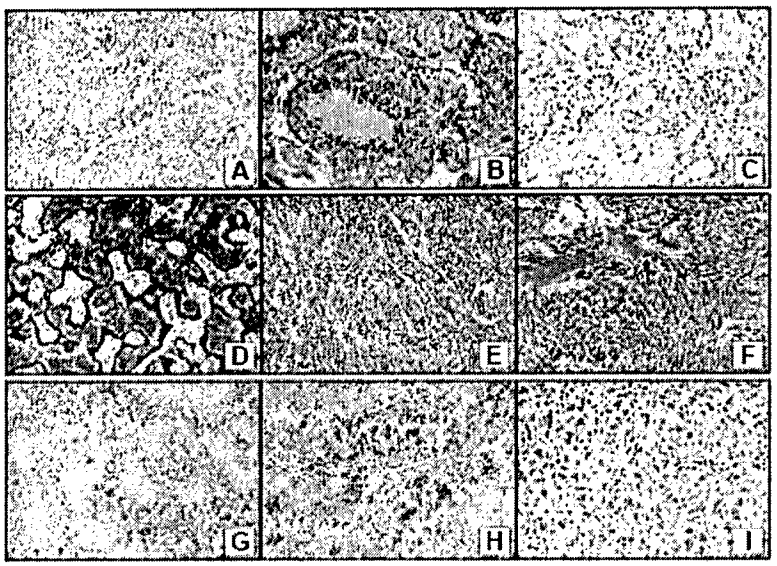
These previous studies, however, lump metastastic tumors as a single entity, while the patterns of dissemination at autopsy suggest that metastatic cancer may better be characterized as a group of diseases rather than a single entity. **Our goal over the last period of the grant was to study the phenotypic and genotypic characteristics of metastatic prostate cancer in detail.** Our data demonstrate that metastatic prostate cancer demonstrates significant heterogeneity, even within the same patient. No consistent differences were found between bone and soft tissue sites that could explain the predilection of prostate cancer cells to metastasize to bone. This suggests that biologic differences leading to the predominant pattern of prostate cancer metastasis to bone may be related to the bone "soil" rather than the prostate cancer "seed". Metastatic hormone refractory PCa has a heterogeneous morphology, immunophenotype, and genotype, demonstrating that "metastatic disease" is a group of diseases even within the same patient. An appreciation of this heterogeneity is critical to evaluating diagnostic and prognostic biomarkers as well as designing therapeutic targets for advanced disease. Further investigation of the bone versus soft tissue stroma in cancer versus normal tissue is warranted. We believe detailed characterization of the heterogeneous phenotypic spectrum of end stage metastatic PCa will guide future molecular studies on metastatic disease, as well as provide a framework for identifying subtypes that may respond better to novel therapeutics.

Body:

Between September 1996 and November 2003, 30 rapid autopsies were performed on men who died of advanced hormone refractory PCa. The median age at time of death was 71 years (range 53-84 years). Twenty eight men were initially diagnosed with clinically localized prostate cancer but developed widely disseminated disease after 5-10 years. Eight men underwent radical prostatectomy before receiving additional treatment and seventeen men received external beam radiation as their primary treatment. Twenty-eight men received combination chemotherapy and all underwent hormonal manipulation. Initial Gleason score was documented in 20 of 30 men and ranged from 4 to 10. The time patients were treated with androgen deprivation ranged from 0 to 144 months. Patients survived in the hormone refractory state (measured as time from first chemotherapy) from 0 to 61 months with a hormone refractory median survival of 14.5 months. Widely disseminated PCa was found in most cases. Bone (83%) was the commonest site of metastasis, followed by liver (66%), lymph nodes (63%), lungs (50%), soft tissues (40%), dura (26%), and adrenal glands (23%). A summary of the percentage distribution pattern of PCa metastasis is presented in Figure 1. Six cases (20%) had predominantly skeleton system involvement, however, no cases presented with only bone involvement. Five cases (16%) presented with only visceral organ involvement (liver and/or lymph node) without grossly observable evidence of bone involvement. The prostate gland was present in 70% (21/30) of cases, of these 6 cases demonstrated androgen therapy effect as has been previously described in primary PCa [12]. Residual tumor was identified in 20/21 cases, eight cases had extensive disease with involvement of bladder and pelvic soft tissues, while 12 had low volume of disease. Review of the histological appearance of these metastatic prostate cancers revealed heterogeneous patterns with architectural patterns similar to primary prostate cancers as described by Gleason [13]. Although Gleason grading in a clinical setting is not recommended for metastatic tumors, we nevertheless applied the Gleason grades in order to use a system that is well known to practicing pathologists [14]. Four cases had uniform tumor morphology at all sites, growing in solid sheets and nests with or without comedonecrosis, resembling Gleason grade 5 (Fig 2A-B). Three cases demonstrated uniform morphology at all sites with tumors growing in a confluent cribriform glandular pattern resembling Gleason grade 4 (Fig 2C). The majority of cases (18 cases) had tumors with mixture of above two growth patterns resembling mixture of Gleason grades 4 and 5. Three cases demonstrated neuroendocrine (NE) differentiation characterized by tumor growing in nests, trabaculae and ribbons with uniform round nuclei, high N: C ratio and salt and pepper chromatin



(Fig 2D-E). Only 1 of these 3 cases was seen as pure growth pattern, while in remaining 2 cases NE differentiation was seen in combination with Gleason grade 5 pattern. Two cases had tumors with small cell carcinoma, similar to oat cell carcinoma of the lung, characterized by spindling, crushing and molding of the nuclei and high mitotic activity (Fig 2F). Three cases had well-formed glands resembling Gleason grade 3 at some metastatic sites in addition to other growth patterns (3 cases)(Fig 2G). In one case metastatic tumors demonstrated poor cohesion with an undifferentiated growth pattern (Fig 2H) and another case demonstrated focal signet ring cell differentiation (Fig 2I) in addition to growth pattern of Gleason grade 5. The wide range of these histological patterns was not associated with a particular organ site.



A tissue microarray (TMA) was constructed from all 30 autopsies to represent all PCa metastasis sites and prostate (if present) [15]. Three cores (0.6 mm in diameter) were taken from each representative tissue block. This TMA was immunostained for a variety of tumor markers, including PSA, Androgen Receptor (AR), Chromogranin (CGA), Synaptophysin (SYN), α-MethyAcylCoenzyme Racemase (AMACR), and the proliferation marker MIB-1. Immunohistochemistry (IHC) was performed using standard avidin-biotin complex protocol. For PSA, CGA, SYN, AMACR and MIB-1 antibodies, antigen retrieval was performed in citrate buffer, at pH 6.0 and for AR; antigen retrieval was performed using EDTA at pH 8.0 in a pressure cooker. The primary antibodies and dilution were as followings: Polyclonal PSA (Dako cytomation, Carpentaria, CA) 1:2000; Monoclonal AR-clone AR 441 (Neo Markers, Fremont, CA) 1:50, CGA (Biogenex, San Ramon, CA) 1:160; SYN (Biogenex, San Ramon, CA) 1:600; AMACR (generated from denatured recombinant antigen to AMACR) 1:5000, and MIB-1 (Dako corporation, Carpentaria, CA) 1:100. The slides were evaluated for adequacy using a standard bright field microscope. Digital images were then acquired using the BLISS Imaging System (Bacus Laboratory, Lombard, IL). Immunohistochemistry evaluation was carried out using Chromavision ACIS II version (Chromavision Medical Systems, Inc) [16]. The ACIS uses pre-programmed advanced color detection software that measures immunohistochemical stains intensity (range 0-255) and percentage expression (0-100%). Because the system has no means of distinguishing tumor tissue from non-cancerous tissue, all images were reviewed by one of the study pathologists. Tumor tissue was electronically circled on the computer screen and only those areas were used to measure

the percentage of the circled cells staining positive for each of the tested markers (0-100%). The final data was recorded in a Microsoft Excel datasheet and used for statistical analysis.

The results are summarized in Table 1. The data presents the median percent staining with the range of the immunomarkers across all patients according to tissue site as present on the tissue microarray. All of the immunomarkers demonstrated significant heterogeneity across disease sites. PSA expression was seen to vary for the percentage of PSA positive cells with median expression of 39.3, range 0.3 to 99.44, standard error 34.5 (Table 1, 2). Consistent with its role as a transcription factor, AR was localized in nuclei. Androgen Receptor expression varied across tumor samples with 31% (83 of 265) tumor samples expressing >50% AR and 41.5% (100/265) expressing <10% AR. Overall expression of AR was down regulated with median AR expression of 20.04% (range, 0-100%, standard error 34.28) (Table 1, 2). The intensity of AMACR expression varied amongst the metastatic tumors, the median percentage of positive tumor cells was 8.18% (range 0.19-71.97%, standard error 14.58). The median MIB-1 expression was 4.55% (range 0.25%-26%, standard error 4.61). CGA and SYN were infrequently observed in these metastatic PCa cases. The median percentage of metastatic tumor cells demonstrating either CGA or SYN protein expression was 2.23% (range 0.21-62.51, standard error of 7.55) and 0.83% (range 0-49.11%, standard error 8.21), respectively. Cases with NE/small cell morphology usually demonstrated NE expression.

Having demonstrated the marked variation of immunomarkers in different organ sites and patients, we next investigated the expression of AR and PSA in tissue sites within the same patient. Table 2 demonstrates the results for 4 different representative patients. Results are present as the median % staining. A range is given if there was more than one sample from that given site in a patient. All of the patients demonstrated marked differences in AR expression between different tissue sites (2-50 fold). There was no pattern to the differences in AR expression between the different organ sites that could be distinguished and this was true across all patients. Perhaps most striking was that several patients demonstrated a high amount of AR staining despite the fact that they were no longer responding to androgen deprivation therapy. PSA expression also varied widely between tissue sites and sometimes, even within the same tissue site of a patient. For example, Case # 30 demonstrated a range of PSA expression from median 3.03 in the prostate to a median of 60.61 in the bone, but the range of expression in the bone itself ranged from 14.67 – 77.86.

Table 1: Demonstration of the immunophenotype of hormone refractory metastatic prostate cancers in 30 rapid autopsy cases

Median % staining (± standard error) of all metastatic samples from 30 rapid autopsy cases evaluated with different immunomarkers

ID	# Met sites	AR	AMACR	SYNAP	MIB1	CGA	PSA	MORPHOLOGY	Time from Chemotherapy to death (months)
1	5	3.6 ± 2.8	17.7 ± 2.3	0.7 ± 0.1	5 ± 0.6	1.8 ± 3.8	79.6 ± 8.7	GP 5+4+NE	26
2	2	4.2 ± 2.5	7.5 ± 1.0	0.6 ± 3.3	8.4 ± 0.4	3.9 ± 1.6	24.1 ± 4.6	NE	21
3	8	44.3 ± 10.8	7.7 ± 2.1	0.2 ± 1.9	4.8 ± 0.9	1.5 ± 0.8	62.7 ± 14.4	GP4+3	20
4	2	23 ± 4.1	2.5 ± 0.3	0.8 ± 1.0	1.6 ± 0.3	0.8 ± 0.5	28.1 ± 5.0	GP 5	13
5	5	21.8 ± 2	10.7 ± 0.7	0.3 ± 1.5	3.3 ± 0.3	3 ± 0.4	16.5 ± 2.8	GP 5	7
6	3	14.1 ± 3.7	30.8 ± 2.9	3.8 ± 1.0	1.3 ± 0.3	3.9 ± 0.6	49 ± 6.3	GP 5+4	2
7	2	NA	1.9 ± 0.3	20.7 ± 2.2	2.8 ± 0.3	8.4 ± 0.7	73.8 ± 1.7	GP 4	34
8	3	8.3 ± 0.0	37.7 ± 3.7	1.4 ± 0.0	7.4 ± 0.2	13.9 ± 0.2	38 ± 6.1	GP 5+4	16
9	1	20 ± 1.2	30.4 ± 2.5	12 ± 0.6	3.4 ± 0.1	5.1 ± 0.1	66.6 ± 1.7	GP 4+3	21
10	3	16.9 ± 2.6	15.6 ± 2.2	19.9 ± 1.1	6.5 ± 0.7	10 ± 0.4	31.1 ± 4.0	GP 5+4	12
11	1	NA	11.4 ± 0.5	7 ± 0	14.3 ± 1.9	2.4 ± 0.1	3.3 ± 0.1	Undifferentiated	5
12	3	0.3 ± 0.2	2.4 ± 0.2	0.4 ± 0.1	2.1 ± 0.3	1.1 ± 0.1	61.9 ± 0.2	GP 5+4	19
13	4	NA	0.6 ± 0.1	0.4 ± 0.2	5.2 ± 1.2	2 ± 0.5	49.8 ± 2.8	GP 5+4+NE	13
14	6	0.7 ± 2.7	23.9 ± 4.9	0.7 ± 2.1	2 ± 0.6	1.2 ± 1.3	83.5 ± 10.1	GP 5+4	17
15	2	56.3 ± 2.8	17.2 ± 4	0.4 ± 0.2	1.7 ± 0.7	0.7 ± 0.3	98.1 ± 2.8	GP 5+4	5
16	4	85.6 ± 4.7	16 ± 2.5	0.7 ± 0.2	14.8 ± 1.3	1.5 ± 0.2	96.9 ± 2.2	GP 5	14
17	4	0.4 ± 0.2	5.7 ± 2.4	8.1 ± 2.4	4.9 ± 0.5	4.1 ± 1.2	2 ± 2.7	GP 5+4+3	11
18	2	75 ± 5.4	3.8 ± 0.2	0.2 ± 0.01	5.1 ± 0.2	1.7 ± 1.0	58.7 ± 1.7	GP 4	14
19	3	43 ± 7	4.1 ± 2.7	2.3 ± 1.0	4.2 ± 0.4	0.9 ± 0.7	87.1 ± 2.0	GP 5	28
20	4	23.9 ± 5	0.4 ± 0.1	0.4 ± 1.5	2.3 ± 0.2	8.8 ± 1.2	2.1 ± 2.6	GP 5+4	14
21	3	0.2 ± 1.3	3.1 ± 0.3	0.9 ± 0.9	1.8 ± 0.3	1.9 ± 0.6	51.2 ± 4.4	GP 5+4	30
22	4	0.3 ± 1.3	3.6 ± 0.9	1.6 ± 4.5	8.8 ± 1.6	5.5 ± 1.6	4.1 ± 2.8	Small cell	9
23	3	52.4 ± 0.4	22.7 ± 3.9	7.8 ± 1.6	3.2 ± 1.0	5 ± 1.1	58.1 ± 5.1	GP 5+4	45
24	6	4.4 ± 3.6	8.2 ± 1.2	3.4 ± 3.4	7.5 ± 2.0	18 ± 4.6	10 ± 10.0	Small cell	9
25	2	32.9 ± 4.2	10.5 ± 0.6	1.2 ± 0.3	11.9 ± 0.1	1.5 ± 0.1	3.3 ± 6.3	GP 5+4	0
26	5	21.7 ± 1.9	15.6 ± 4.7	1.8 ± 1.9	7.8 ± 1.9	3.6 ± 0.7	19.9 ± 5.3	GP 5+4	35
27	1	29.2 ± 3.7	8 ± 0.3	6.9 ± 0.6	2.3 ± 0.1	7.3 ± 0.9	40.6 ± 2.1	GP 5+4	15
28	4	83.4 ± 8.3	16.7 ± 4.3	0.2 ± 1.6	5.9 ± 1.0	1.1 ± 0.8	64.5 ± 6.5	GP 5+4	61
29	1	47.3 ± 0.0	13.3 ± 0	15.3 ± 1.6	4.8 ± 0.3	6.8 ± 0.0	33.4 ± 2.5	GP 5+4	8
30	7	87 ± 7.7	5.8 ± 2.7	0.5 ± 1.9	4.6 ± 1.0	1.9 ± 0.9	8.5 ± 5.8	GP 5+4	16

SYNAP=Synaptophysin, CGA=Chromogranin, AR=Androgen Receptor, GP=Gleason Pattern, NE=Neuroendocrine

TABLES 2 a and b: Demonstration of heterogeneity in PSA and Androgen Receptor protein expression of metastatic sites in the same patient

Table 2a. Androgen receptor staining across metastatic sites for rapid autopsy case # 3, 14, 24, 30.

Metastatic Tissue	RA case # 3	RA case # 14	RA case # 24	RA case # 30
		Median % staining of AR (range)		
Adrenal	74.28 (64.4 , 78.93)	--	--	--
Bone	22.66 (22.66 , 22.66)	--	--	81.07 (75.74 , 86.4)
Bladder	--	--	--	89.34 (88.54 , 96.65)
Dura	33.59 (5.17 , 62.01)	--	5.45 (1.86 , 9.04)	--
Liver	46.22 (18.8 , 83.1)	0.48 (0.41 , 1.78)	2.28 (0.05 , 7.14)	--
Lung	23.67 (15.41 , 27.4)	0.18 (0.1 , 0.22)	13.03 (5.64 , 41.95)	96.89 (85.49 , 99.56)
Lymph Node	70.81 (40.78 , 94.65)	--	0.06 (0.06 , 0.06)	99.38 (99.01 , 99.58)
Meninges	--	0.73 (0.64 , 0.81)	0.01 (0 , 0.01)	68.93 (18.31 , 72.12)
Prostate	12.23 (3.7 , 13.61)	1.81 (1.29 , 2.33)	--	47.83 (16.3 , 87.04)
Sofe Tissue	--	19.99 (0.49 , 29.08)	--	--
Testis	10.7 (10.7 , 10.7)	--	--	--

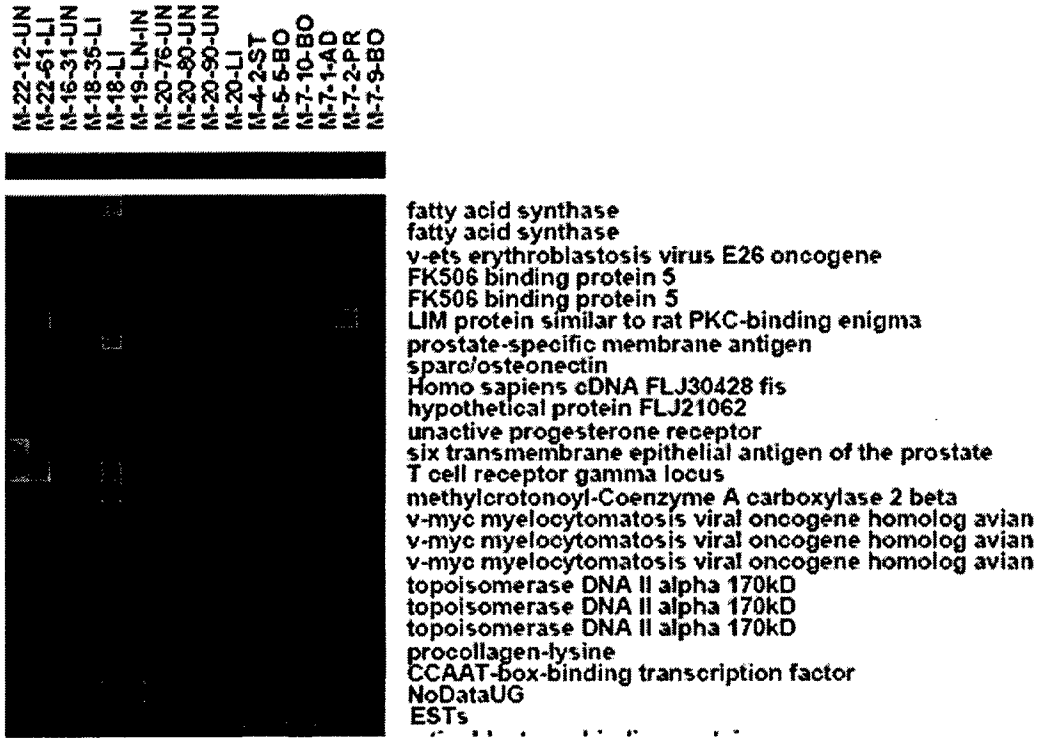
Note: There is one metastatic site less for case # 14 and 24 each of AR staining due to missing values.

Table 2b. PSA staining across metastatic tissue for rapid autopsy case # 3, 14, 24, 30.

Metastatic Tissue	RA case # 3	RA case # 14	RA case # 24	WA case # 30
		Median % staining of PSA (range)		
Adrenal	98.54 (98.42 , 98.89)	--	--	--
Bone	47.86 (44.63 , 51.08)	64.92 (57.03 , 72.82)	61.62 (61.62 , 61.62)	60.61 (14.67 , 77.86)
Bladder	--	--	--	2.84 (2.02 , 3.61)
Dura	47.27 (6.25 , 88.3)	--	2.27 (1.43 , 2.33)	5.84 (5.84 , 5.84)
Liver	39.41 (5.72 , 77.58)	96.03 (94.14 , 97.58)	20.14 (5.85 , 39.3)	--
Lung	28.93 (7.87 , 49.09)	83.42 (12.43 , 97.61)	2.74 (1.47 , 95.45)	3.59 (0.34 , 14.07)
Lymph Node	97 (86.18 , 98.29)	--	8.57 (8.57 , 8.57)	9.1 (8.53 , 13.27)
Meninges	--	41.65 (31.83 , 51.47)	--	--
Prostate	61.67 (57.49 , 63.7)	4.27 (3.59 , 4.95)	13.05 (11.95 , 14.16)	3.03 (1.2 , 5.22)
Soft Tissue	--	93.77 (83.49 , 98.61)	--	20.91 (3.91 , 39.04)
Testis	97.79 (97.79 , 97.79)	--	--	--

We next sought to determine if the heterogeneity in histology and phenotype was mirrored by heterogeneity in gene expression. Using expression array data that we have previously generated [6, 7], we performed hierarchical clustering of the 16 metastatic samples from 6 cases in the rapid autopsy series. The metastatic adenocarcinoma samples were highly heterogenous (figure 3). Only two metastases from a patient with small cell histology demonstrated comparable cDNA expression results (figure 3). Utilizing the Oncomine database, we found that these tumors exhibited a similar expression pattern as small cell lung cancer.

Figure 3. cDNA expression heat map from tumor metastases from different patients and different metastatic sites. Expression patterns did not correlate between bone and soft tissue sites.



We, as well as others, have identified several genes that are differentially expressed in primary versus metastatic disease [6, 7, 17]. By definition, these analyses are designed to find potential similarities between samples at a similar disease stage and compare the two groups. While these studies have identified several potential biomarkers, they were also remarkable for the amount of heterogeneity and overlap between primary and metastatic samples. To date, metastatic prostate tissue samples from the same patient and between patients have not been systematically been analyzed in an attempt to quantify this heterogeneity and determine if it may be important. Our data demonstrate that there are significant differences in the genotype and phenotype of metastatic prostate cancer between patients and within the same patient. There does not appear to be a correlation between bone and soft tissue sites in this extensive study as compared to our earlier preliminary data.

Key research accomplishments:

- Demonstrated that there is no consistent gene expression pattern that differentiates bone and soft tissue metastases in prostate cancer.
- Demonstrated that there are no consistent phenotypic expression patterns that differentiate bone and soft tissue metastases in prostate cancer.

Reportable outcomes (last grant period):

Manuscripts:

Rajal B. Shah, M.D., Rohit Mehra, M.D., Arul M. Chinnaiyan, M.D., PhD, Ronglai Shen, M.S., Debashish Ghosh, PhD, Ming Zhou, M.D., PhD, Gary R. MacVicar, M.D., Soorynarayana Varambally, PhD, Jason Harwood, B.S., Tarek A. Bismar, M.D., Robert Kim, B.S., Mark A. Rubin, M.D., and Kenneth J. Pienta, M.D. Androgen Independent Prostate Cancer is a Heterogeneous Group of Diseases: Lessons from a Rapid Autopsy Program. Cancer Research, IN PRESS.

Tantivejkul K, Kalikin LM, Pienta KJ. Dynamic process of prostate cancer metastasis to bone. J Cell Biochem. 2004 Mar 1;91(4):706-17.

Conclusions:

Stephen Paget first postulated a "seed" versus "soil" hypothesis to describe the metastatic process, believing that metastasis was dependent on both properties inherent in the cancer cell as well as the microenvironment of the metastatic site. The purpose of this grant was to explore the properties inherent in the cancer cells themselves by investigating the phenotypic and genotypic traits of metastatic prostate cancer from various sites as well as cell lines derived from various sites. Our data demonstrated differences in the ability to cells derived from bone metastases as compared to soft tissue metastases to adhere to bone derived endothelium. Our move to human tissues over the last grant period to investigate gene expression in detail has demonstrated no clear gene expression pattern that leads to bone versus soft tissue metastasis. This leads us to ask the next question which is what aspects of the target organ microenvironment are important for preferential metastasis. This will be the subject of future studies.

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The reviewer asked for documentation of animal and human use approval during the last two years of the grant:

Animal Approval: UCUCA 8434, approval appended
Human use IRB: IRB approval 1998-0157, approval appended

The reviewer asked for the cited articles to be appended: articles appended

The reviewer has asked for a new statement of work (SOW) that better reflects the experiments that evolved as the grant progressed. The SOW has been delineated below:

Statement of Work

Biological Differences Between Prostate Cancer Cells That Metastasize to Bone versus Soft Tissue Sites

Task 1. To investigate how the bone microenvironment modulates growth and adhesion of VCap versus DuCaP (Months 1-36)

1A: Investigate the effects of known bone marrow cytokines on the growth of VCaP and DuCaP. We will investigate the effects of transferring, TGF-beta, OPG, OPN, OCN, BMPs, PDGF, bFGF, and other factors by measuring the effects of these cytokines on cell doubling time in a dose dependent manner (Months 1-12).

1B: Investigate the effects of bone extracellular matrix components on the growth of VCaP versus DuCaP cells. We will investigate intact bone marrow matrix as well as individual components of the matrix including fibronectin, laminin, and collagen I by measuring the effects of these matrix components on cell doubling time in a dose dependent manner. These will then be done in combination with the important cytokines that modulate growth as determined in 1A (Months 13-24).

1C: Investigate the adhesion of VCaP versus DuCaP to human bone marrow endothelium (HBME). We will investigate differential binding of the two prostate cancer cell lines to HBME. We will investigate whether cytokines, such as TGF-β1, bFGF, IGF type I, IGF type II, EGF, IL-6, osteopontin, BMO 4, BMP 5, and BMP 6, also modulate their adhesion. We will investigate the effect of plating the HBME cells on various bone matrix components up or down regulate the adhesion of the two prostate cancer cell lines (Months 25-36).

Task 2. To investigate the differential gene expression of VCaP versus DuCaP cells by cDNA microarrays to identify specific gene products important in the metastasis of prostate cancer to bone.

2A: **Gene profiling.** We will investigate the molecular profile of VCaP and DUCaP cells utilizing cDNA microarray technology. A 9,978 element human cDNA microarray composed of approximately 4000 known, named genes from the Research Genetics human cDNA clone set, 5000 ESTs, 500 control elements, and 500 specific cancer and apoptosis related genes will e utilized. In addition to VCaP and DuCaP, samples will be initially taken from the cell lines PC-3, DU145, and LNCaP. Genes that are overexpressed or underexpressed will be confirmed by Northern analysis (Months 1-12).

2B: **Characterization of proteins.** We will characterize known or unknown gene products that are differentially expressed between VCaP and DuCaP. Studies will be targeted to characterizing identified proteins by sequence analysis, genbank searches, antibody development, affinity chromatography, and subceclular localization (Months 13-36).

Task 3. (NEW) Development of a preclinical model to study prostate cancer metastasis in SCID mice.

3A: Transfection and testing of cell lines. VCaP and DuCaP cell lines will be stably transfected with a retroviral vector containing luciferase. These will then be injected intracardiac into SCID mice and the pattern of metastases will be tracked and documented (Months 12-24).

Task 4. (NEW) Investigation of tumor cell heterogeneity in metastatic prostate cancer.

4A: We will investigate samples from 30 rapid autopsies of men who died of advanced prostate cancer by immunohistochemistry of tissue microarrays. Soft tissue and bone metastases between patients as well as from the same patient will be studied (<100 metastases). TMA's will be stained for expression of PSA, AR, chromagranin, and other markers. Preliminary gene expression studies between metastases will also be studied by microarrays (Months 24-36).

University of Michigan Medical School

The University of Michigan Medical School
Institutional Review Board for Human Subject Research (IRBMED)
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NOTICE OF OUTCOME OF REVIEW OF HUMAN SUBJECT RESEARCH

FAX TO: 7-9480

Pienta,Kenneth (Principal Investigator) •• IRBMED #: 1998-0157
Project Title: Analysis of Genetic Changes in Human Prostate Cancer
Sponsor & Identifier Code: Health and Human Services, Department of-National Institutes of Health (SPORE): 1 P50 CA69568
Submit Date: Sep 01, 2004 •• **Receipt Date:** Sep 01, 2004
Project Type: No direct involvement of human subjects (retrospective): Research using human data or biological specimens.
Supporting Documents: Waiver of Informed Consent
Application Type: Scheduled-Continuation Review
FDA-Regulated Test Articles: No test articles used.
Vulnerable Subjects: None
Informed Consent Process: Waiver/alteration of consent
HIPAA Compliant: Yes (PHI as described in application or protocol) •• **Review Type:** Expedited(No Vote)
Risk Level: No More Than Minimal
Outcome: Approved/Acknowledgement •• **Decision Date:** Sep 16, 2004
Expiration Date of Project Approval: Sep 15, 2005

Information on the project and the outcome of the review by the IRBMED appear in the descriptive paragraph above. The content of the submitted material conforms to relevant regulations of the United States Government and the University of Michigan. If the descriptive paragraph indicates that a "Comprehensive Written" informed consent process is to be implemented, all copies of the consent document are required to display the following information in the descriptive paragraph: [1] IRBMED Archive Number. [2] Approval Date of Most Recent Version of Consent Document. [3] Expiration Date of Project Approval. If the descriptive paragraph acknowledges that the project has been terminated, all activity involving human subjects will have come to an end, and will not resume unless reviewed and approved by the IRBMED as a new or extension project.

The investigators are required to report to the IRBMED [1] planned changes in any aspect of the study, and do not implement any change without receiving approval, except to eliminate immediate hazard to subjects, [2] any serious or unexpected adverse events, and [3] any new information on the project that may adversely influence the risk/benefit ratio.

The investigators are responsible for applying to the IRBMED to receive Scheduled-Continuation Review and Approval of the project about eight weeks prior to the "Expiration Date of Project Approval" shown in the descriptive paragraph. In case IRBMED approval is not secured prior to Expiration Date, subject recruitment activity will cease, and no research interventions will be administered to the research subjects except to eliminate immediate hazard.

A list of IRBMED members is available at the IRBMED Internet web site ("Membership Roster (IRBMED)"). This Notice of Outcome document and the membership roster may be submitted to sponsors of the research.

Note: If this research study will take place in the General Clinical Research Center (GCRC) please remember to send the GCRC a copy of all IRBMED approval letters and approved consent forms. Send to: GCRC, A7119 UH, Box 0108 or fax to 936-4024. GCRC phone: 936-9090. CGRC Website: http://www.med.umich.edu/gcrc/

John G. Weg, M.D.
Professor Emeritus, Internal Medicine
Co-Chair, IRBMED

Copies to:
Principal Investigator
Medical School Assoc. Dean for Research & Graduate Studies
Division of Research & Development Administration

[IRBMED April 2003]

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February 19, 2004

UCUCA ANNUAL REVIEW FORM

APPLICATION TO USE VERTEBRATE ANIMALS IN RESEARCH, TESTING OR INSTRUCTION

The regulations of the Federal Animal Welfare Act require the University of Michigan Committee on Use and Care of Animals (UCUCA) to review annually all research, teaching, and testing that utilizes animals. The Annual Review ensures that the information provided in your approved Application to Use Vertebrate Animals (Form 8225) is still accurate and in compliance with University policy.

Please complete, sign and return this form to the UCUCA Office **within 30 days of the date of this notice**. Contact the UCUCA Office at 763-8028 if you require further information.

PLEASE NOTE: FAILURE TO COMPLY WITH THIS REQUIREMENT IS A VIOLATION OF FEDERAL LAW. THIS FORM MUST BE COMPLETED AND RETURNED TO THE UCUCA OFFICE BY THE DATE INDICATED ABOVE TO AVOID INACTIVATION OF UCUCA APPROVAL FOR THE REFERENCED PROJECT.

Title(s): **SPORE in Prostate Cancer, Biological Differences Between Prostate Cancer Cells that Metast, CaPCURE Therapy Consortium**

Principal Investigator:	**Pienta, Kenneth J**
Department:	**IM**
Address:	**7436 CCGC 0946**
Sponsor(s):	**National Institutes of Health, Department of Defense, CaP Cure**
Approval/Application Number:	**8434**
Approval Date:	**5/28/2002**

P Planned continuation of the study as originally described.

____ Planned continuation of study with changes.

PLEASE NOTE IMPORTANT INFORMATION BELOW

____ Study discontinued.

Signature: _____ Date: 2/25/04.
(Principal Investigator/Course Director)

Important Information

[redacted]

RETURN BOTH FORMS TO: UCUCA OFFICE, 3502 ARF 0614

[CANCER RESEARCH 64, 9209–9216, December 15, 2004]

Androgen-Independent Prostate Cancer Is a Heterogeneous Group of Diseases: Lessons from a Rapid Autopsy Program

Rajal B. Shah,[1,3] Rohit Mehra,[1] Arul M. Chinnaiyan,[1,3] Ronglai Shen,[4] Debashis Ghosh,[4] Ming Zhou,[1] Gary R. MacVicar,[2] Soorynarayana Varambally,[1] Jason Harwood,[1] Tarek A. Bismar,[5] Robert Kim,[5] Mark A. Rubin,[5,6,7] and Kenneth J. Pienta[2,3]

Departments of [1]Pathology, [2]Medical Oncology, [3]Urology, and [4]Biostatistics, University of Michigan School of Medicine, Ann Arbor, Michigan; [5]Department of Pathology, Brigham and Women's Hospital, Boston, Massachusetts; [6]Department of Pathology, Dana Farber Cancer Institute, Boston, Massachusetts; and [7]Department of Pathology, Harvard Medical School, Boston, Massachusetts

ABSTRACT

Understanding the biology of prostate cancer metastasis has been limited by the lack of tissue for study. We studied the clinical data, distribution of prostate cancer involvement, morphology, immunophenotypes, and gene expression from 30 rapid autopsies of men who died of hormone-refractory prostate cancer. A tissue microarray was constructed and quantitatively evaluated for expression of prostate-specific antigen, androgen receptor, chromogranin, synaptophysin, MIB-1, and α-methylacylCoA-racemase markers. Hierarchical clustering of 16 rapid autopsy tumor samples was performed to evaluate the cDNA expression pattern associated with the morphology. Comparisons were made between patients as well as within the same patient. Metastatic hormone-refractory prostate cancer has a heterogeneous morphology, immunophenotype, and genotype, demonstrating that "metastatic disease" is a group of diseases even within the same patient. An appreciation of this heterogeneity is critical to evaluating diagnostic and prognostic biomarkers as well as to designing therapeutic targets for advanced disease.

INTRODUCTION

In the United States, prostate cancer remains the most common solid tumor malignancy in men, causing ~30,000 deaths in 2004 (1). With the goal of trying to better understand the biology of prostate tumorigenesis and metastasis, we have developed a rapid autopsy program to collect tumors from multiple sites including solid organs and bone to perform molecular studies to better delineate prostate cancer progression (2). Our group has used these samples with a combination of cDNA expression and tissue microarray analysis approaches to identify novel prostate cancer biomarkers, including Enhancer of Zeste homolog 2 (EZH2), Metastasis associated 1 (MTA1), PIM1, and α-methylacyl-CoA racemase (AMACR), with a supervised analysis comparing androgen-independent to localized prostate cancer samples (3–7).

These previous studies, however, lump metastatic tumors as a single entity, whereas the patterns of dissemination at autopsy suggest that metastatic cancer may better be characterized as a group of diseases rather than a single entity. Our goal of the present study was to study the phenotypic characteristics of metastatic prostate cancer in detail. Our data indicate that metastatic prostate cancer demonstrates substantial heterogeneity, even within the same patient. We believe detailed characterization of the heterogeneous phenotypic spectrum of end stage metastatic prostate cancer will guide future molecular studies on metastatic disease, as well as provide a framework for identifying subtypes that may respond better to novel therapeutics.

MATERIALS AND METHODS

Rapid Autopsy Tissue Procurement Protocol. The rapid autopsy program was approved by the Institutional Review Board of University of Michigan and supported by Specialized Program of Research Excellence in Prostate Cancer (National Cancer Institute grant CA69568) and the Prostate Cancer Foundation. Patients were identified with hormone-refractory prostate cancer by the Medical Oncology Service of the Comprehensive Cancer Center at the University of Michigan Hospitals as described previously (2). The clinical information obtained for each patient included age at diagnosis, age at death, months to death after diagnosis, initial Gleason score, time on hormonal therapy only, and type of primary and systemic therapy. These autopsies have been referred to as "rapid" or "warm" because of the short time interval (average 3 hours) between patient death and starting the autopsy.

Pathology Data. Pathology data included detailed histologic evaluation of prostate cancer at metastatic sites, location and systemic distribution pattern of metastasis at both osseous and nonosseous organ sites, and histology of the prostate in event of no previous radical prostatectomy. Microscopic evaluation of all slides from each tumor site was evaluated by the study pathologists (R. B. S., R. M., M. A. R.).

Construction of Tissue Microarray and Immunohistochemistry. A tissue microarray was constructed from all 30 autopsies to represent all of the prostate cancer metastasis sites and the prostate (if present). Three cores (0.6 mm in diameter) were taken from each representative tissue block. The tissue microarray construction protocol has been described previously (8–10). This tissue microarray was immunostained for a variety of tumor markers, including prostate-specific antigen (PSA), androgen receptor (AR), chromogranin (CGA), synaptophysin (SYN), α-methylacylCoA-racemase (AMACR), and the proliferation marker MIB-1. Immunohistochemistry was performed with standard avidin-biotin complex protocol. For PSA, CGA, SYN, AMACR and MIB-1 antibodies, antigen retrieval was performed in citrate buffer at pH 6.0; and for AR, antigen retrieval was performed with EDTA at pH 8.0 in a pressure cooker. The primary antibodies and dilution were as followings: polyclonal PSA (Dako Cytomation, Carpenteria, CA) 1:2000; monoclonal AR-clone AR 441 (NeoMarkers, Fremont, CA) 1:50; CGA (Biogenex, San Ramon, CA) 1:160; SYN (Biogenex) 1:600; AMACR (generated from denatured recombinant antigen to AMACR) 1:5000; and MIB-1 (Dako Corporation, Carpentaria, CA) 1:100. The slides were evaluated for adequacy with a standard bright field microscope. Digital images were then acquired with the BLISS Imaging System (Bacus Laboratory, Lombard, IL).

Immunohistochemistry evaluation was carried out with Chromavision ACIS II version (Chromavision Medical Systems, Inc., San Juan Capistrano, CA; refs. 11–13). The ACIS uses preprogrammed advanced color detection software that measures immunohistochemical stains intensity (range, 0–255) and percentage expression (0–100%). Because the system has no means of distinguishing tumor tissue from noncancerous tissue, all of the images were reviewed by one of the study pathologists (T. A. B.). Tumor tissue was electronically circled on the computer screen, and only those areas were used to measure the percentage of the circled cells that stained positive for each of

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Note: Presented in part at the United States and Canadian Academy of Pathology Annual Meeting, Vancouver, BC, Canada, March, 2004; M. A. Rubin and K. J. Pienta share senior authorship.

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Table 1 *Clinical characteristics of 30 rapid autopsies*

Case no.	Age at death (year)	Gleason score at initial diagnosis	Treatment *	Months treated with hormones prior to chemotherapy	Months hormone refractory
1	77	9	R, H, C	23	26
2	59	Metastasis †	H, C, X	12	21
3	53	Metastasis †	H, C, X	0	20
4	71	4 ‡	R, H, C	24	13
5	54	9	R, H, C	36	7
6	71	6 ‡	P, R, H, C	30	2
7	65	9	R, H, C	31	34
8	68	Unknown	P, R, H, C	51	16
9	68	7 ‡	P, R, H, C	144	21
10	78	Unknown	R, H, C	24	12
11	67	8	R, H, C	12	5
12	79	7	H, C	42	19
13	71	Unknown	P, R, H, C	120	13
14	76	6	R, H, C	84	17
15	74	10	R, H, C	4	3
16	70	7	P, R, H, C	24	14
17	74	7	H, C, X	21	11
18	61	5 ‡	P, R, H, C	60	14
19	84	7	R, H, C, X	69	28
20	66	9	P, R, H, C, X	65	14
21	64	9	R, H, C	25	30
22	64	8	H, C, X	22	9
23	72	6	R, H, C	27	45
24	76	7	R, H, C, X	13	9
25	66	Unknown	H, X	42	0
26	76	Unknown	R, H, C, X	60	35
27	74	7	R, H, C	80	15
28	86	Unknown	P, H, C	119	61
29	77	8	R, H, C	102	8
30	71	9	H, C, X	13	16

* Treatment regimens: H, hormone ablation by bilateral orchiectomy and/or pharmacological blockage; C, chemotherapy; R, primary or salvage radiation; P, radical prostatectomy; X, palliative radiation.

† Initial presentation as metastasis outside prostate.

‡ Pathology slides not available for review.

the tested markers (0–100%). The final data were recorded in a Microsoft Excel datasheet and were used for statistical analysis.

Microarray Analysis (cDNA). The spotted cDNA microarrays used for the identification of differentially expressed genes in the rapid autopsy series have been previously described (6, 7). The cDNA arrays contained ~5,500 known, named genes from the Research Genetics human cDNA clone set, and >4,400 expressed sequence tags (7). The expression array data were analyzed with a Cluster and TreeView[8] to explore for relationships between samples (14).

Statistical Analysis. Regression tree was fitted with time from chemotherapy to death as response, and each immunohistochemistry marker percentage staining values was dichotomized according to the root node splitter from the fitted tree. Survival time was divided into >14.5 months and <14.5 months to represent good *versus* bad outcome, and 2 × 2 contingency table was generated to test the association between the outcome and maker staining levels. Exact test and linear regression were also performed with statistical software package (SAS Institute Inc., Cary, NC) to measure association between different clinical variables and immunomarkers with survival time.

RESULTS

Clinical Findings from 30 Rapid Autopsy Cases. Between September 1996 and November 2003, 30 rapid autopsies were performed on men who died of advanced hormone-refractory prostate cancer (Table 1). The median age at time of death was 71 years (range, 53–84 years). Twenty-eight men were initially diagnosed with clinically localized prostate cancer but developed widely disseminated disease after 5–10 years. Eight men underwent radical prostatectomy before receiving additional treatment and 17 men received external beam radiation as their primary treatment. Twenty-eight men received combination chemotherapy, and all underwent hormonal manipulation. Initial Gleason score was documented in 20 of 30 men and ranged from 4 to 10. The time that patients were treated with androgen deprivation ranged from 0 to 144 months. Patients survived in the hormone-refractory state (measured as time from first chemotherapy) from 0 to 61 months with a hormone-refractory median survival of 14.5 months.

Rapid Autopsy Findings. Widely disseminated prostate cancer was found in most cases. Bone (83%) was the commonest site of metastasis, followed by liver (66%), lymph nodes (63%), lungs (50%), soft tissues (40%), dura (26%), and adrenal glands (23%). A summary of the percentage distribution pattern of prostate cancer metastasis is presented in Fig. 1*A*. Six cases (20%) had predominantly skeleton system involvement; however, no cases presented with only bone involvement. Five cases (16%) presented with only visceral organ involvement (liver and/or lymph node) without grossly observable evidence of bone involvement. The prostate gland was present in 70% (21 of 30) of cases; of these, 6 cases demonstrated androgen therapy effect as has been previously described in primary prostate cancer (15). Residual tumor was identified in 20 of 21 cases, 8 cases had extensive disease with involvement of bladder and pelvic soft tissues, and 12 had low volume of disease.

Review of the histologic appearance of these metastatic prostate cancers revealed heterogeneous patterns with architectural patterns

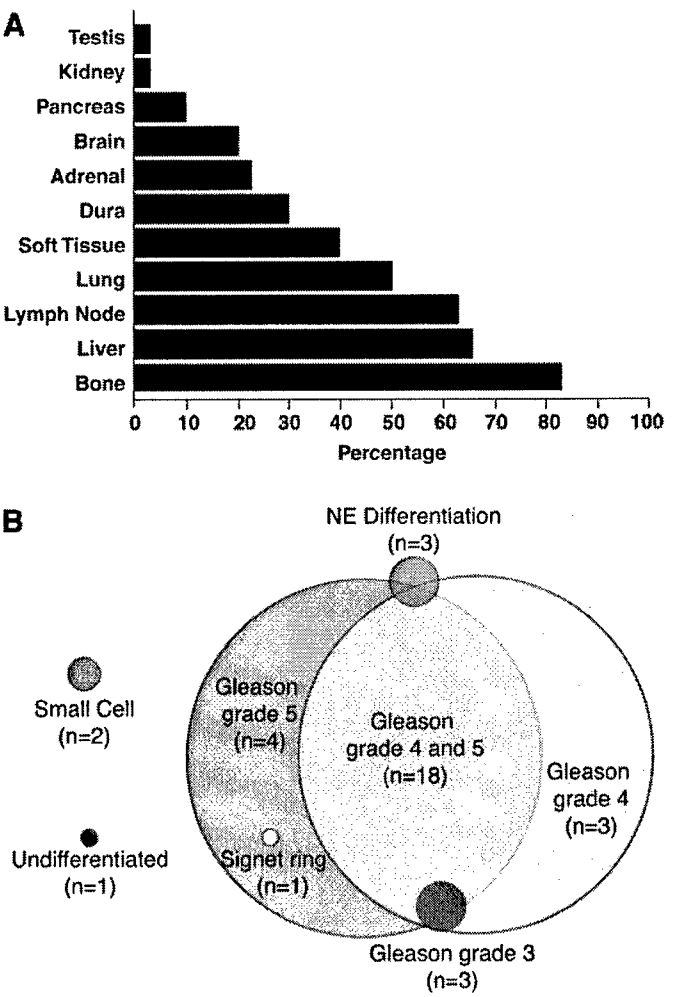

Fig. 1. *A*, distribution of hormone-refractory prostate cancer metastasis from 30 rapid autopsy cases performed at the University of Michigan. *B*, schematic overview of the distribution and overlap of the different histologic patterns identified in the cases from the rapid autopsy hormone-refractory metastatic prostate cancers (*NE*, neuroendocrine).

[8] http://www.microarrays.org.

Table 2 *Demonstration of the immunophenotype of hormone-refractory metastatic (met) prostate cancers in 30 rapid autopsy cases*

ID	No. of met sites	AR	AMACR	SYN	MIB-1	CGA	PSA	Morphology	Time from chemotherapy to death (months)
1	5	3.6 ± 2.8	17.7 ± 2.3	0.7 ± 0.1	5 ± 0.6	1.8 ± 3.8	79.6 ± 8.7	GP 5 + 4+NE	26
2	2	4.2 ± 2.5	7.5 ± 1.0	0.6 ± 3.3	8.4 ± 0.4	3.9 ± 1.6	24.1 ± 4.6	NE	21
3	8	44.3 ± 10.8	7.7 ± 2.1	0.2 ± 1.9	4.8 ± 0.9	1.5 ± 0.8	62.7 ± 14.4	GP4 + 3	20
4	2	23 ± 4.1	2.5 ± 0.3	0.8 ± 1.0	1.6 ± 0.3	0.8 ± 0.5	28.1 ± 5.0	GP 5	13
5	5	21.8 ± 2	10.7 ± 0.7	0.3 ± 1.5	3.3 ± 0.3	3 ± 0.4	16.5 ± 2.8	GP 5	7
6	3	14.1 ± 3.7	30.8 ± 2.9	3.8 ± 1.0	1.3 ± 0.3	3.9 ± 0.6	49 ± 6.3	GP 5 + 4	2
7	2	NA	1.9 ± 0.3	20.7 ± 2.2	2.8 ± 0.3	8.4 ± 0.7	73.8 ± 1.7	GP 4	34
8	3	8.3 ± 0.0	37.7 ± 3.7	1.4 ± 0.0	7.4 ± 0.2	13.9 ± 0.2	38 ± 6.1	GP 5 + 4	16
9	1	20 ± 1.2	30.4 ± 2.5	12 ± 0.6	3.4 ± 0.1	5.1 ± 0.1	66.6 ± 1.7	GP 4 + 3	21
10	3	16.9 ± 2.6	15.6 ± 2.2	19.9 ± 1.1	6.5 ± 0.7	10 ± 0.4	31.1 ± 4.0	GP 5 + 4	12
11	1	NA	11.4 ± 0.5	7 ± 0	14.3 ± 1.9	2.4 ± 0.1	3.3 ± 0.1	Undifferentiated	5
12	3	0.3 ± 0.2	2.4 ± 0.2	0.4 ± 0.1	2.1 ± 0.3	1.1 ± 0.1	61.9 ± 0.2	GP 5 + 4	19
13	4	NA	0.6 ± 0.1	0.4 ± 0.2	5.2 ± 1.2	2 ± 0.5	49.8 ± 2.8	GP 5 + 4+NE	13
14	6	0.7 ± 2.7	23.9 ± 4.9	0.7 ± 2.1	2 ± 0.6	1.2 ± 1.3	83.5 ± 10.1	GP 5 + 4	17
15	2	56.3 ± 2.8	17.2 ± 4	0.4 ± 0.2	1.7 ± 0.7	0.7 ± 0.3	98.1 ± 2.8	GP 5 + 4	5
16	4	85.6 ± 4.7	16 ± 2.5	0.7 ± 0.2	14.8 ± 1.3	1.5 ± 0.2	96.9 ± 2.2	GP 5	14
17	4	0.4 ± 0.2	5.7 ± 2.4	8.1 ± 2.4	4.9 ± 0.5	4.1 ± 1.2	2 ± 2.7	GP 5 + 4+3	11
18	2	75 ± 5.4	3.8 ± 0.2	0.2 ± 0.01	5.1 ± 0.2	1.7 ± 1.0	58.7 ± 1.7	GP 4	14
19	3	43 ± 7	4.1 ± 2.7	2.3 ± 1.0	4.2 ± 0.4	0.9 ± 0.7	87.1 ± 2.0	GP 5	28
20	4	23.9 ± 5	0.4 ± 0.1	0.4 ± 1.5	2.3 ± 0.2	8.8 ± 1.2	2.1 ± 2.6	GP 5 + 4	14
21	3	0.2 ± 1.3	3.1 ± 0.3	0.9 ± 0.9	1.8 ± 0.3	1.9 ± 0.6	51.2 ± 4.4	GP 5 + 4	30
22	4	0.3 ± 1.3	3.6 ± 0.9	1.6 ± 4.5	8.8 ± 1.6	5.5 ± 1.6	4.1 ± 2.8	Small cell	9
23	3	52.4 ± 0.4	22.7 ± 3.9	7.8 ± 1.6	3.2 ± 1.0	5 ± 1.1	58.1 ± 5.1	GP 5 + 4	45
24	6	4.4 ± 3.6	8.2 ± 1.2	3.4 ± 3.4	7.5 ± 2.0	18 ± 4.6	10 ± 10.0	Small cell	9
25	2	32.9 ± 4.2	10.5 ± 0.6	1.2 ± 0.3	11.9 ± 0.1	1.5 ± 0.1	3.3 ± 6.3	GP 5 + 4	0
26	5	21.7 ± 1.9	15.6 ± 4.7	1.8 ± 1.9	7.8 ± 1.9	3.6 ± 0.7	19.9 ± 5.3	GP 5 + 4	35
27	1	29.2 ± 3.7	8 ± 0.3	6.9 ± 0.6	2.3 ± 0.1	7.3 ± 0.9	40.6 ± 2.1	GP 5 + 4	15
28	4	83.4 ± 8.3	16.7 ± 4.3	0.2 ± 1.6	5.9 ± 1.0	1.1 ± 0.8	64.5 ± 6.5	GP 5 + 4	61
29	1	47.3 ± 0.0	13.3 ± 0	15.3 ± 1.6	4.8 ± 0.3	6.8 ± 0.0	33.4 ± 2.5	GP 5 + 4	8
30	7	87 ± 7.7	5.8 ± 2.7	0.5 ± 1.9	4.6 ± 1.0	1.9 ± 0.9	8.5 ± 5.8	GP 5 + 4	16

NOTE. Median % staining (± SEM) of all metastatic samples from 30 rapid autopsy cases evaluated with different immunomarkers.
Abbreviations: ID, identification number; GP, Gleason pattern; NE, neuroendocrine; NA, Not available.

similar to primary prostate cancers as described by Gleason (16). Although Gleason grading in a clinical setting is not recommended for metastatic tumors (17), we nevertheless applied the Gleason grades to use a system that is well known to practicing pathologists. The distribution and overlap of different histologic patterns identified in these cases are schematically demonstrated in Fig. 1*B* and summarized in Table 2. Four cases had uniform tumor morphology at all sites, growing in solid sheets and nests with or without comedonecrosis, resembling Gleason grade 5 (Fig. 2*A* and *B*). Three cases demonstrated uniform morphology at all sites, with tumors growing in a confluent cribriform glandular pattern resembling Gleason grade 4 (Fig. 2*C*). The majority of cases (18 cases) had tumors with a mixture of the above two growth patterns resembling a mixture of Gleason grades 4 and 5. Three cases demonstrated neuroendocrine differenti-

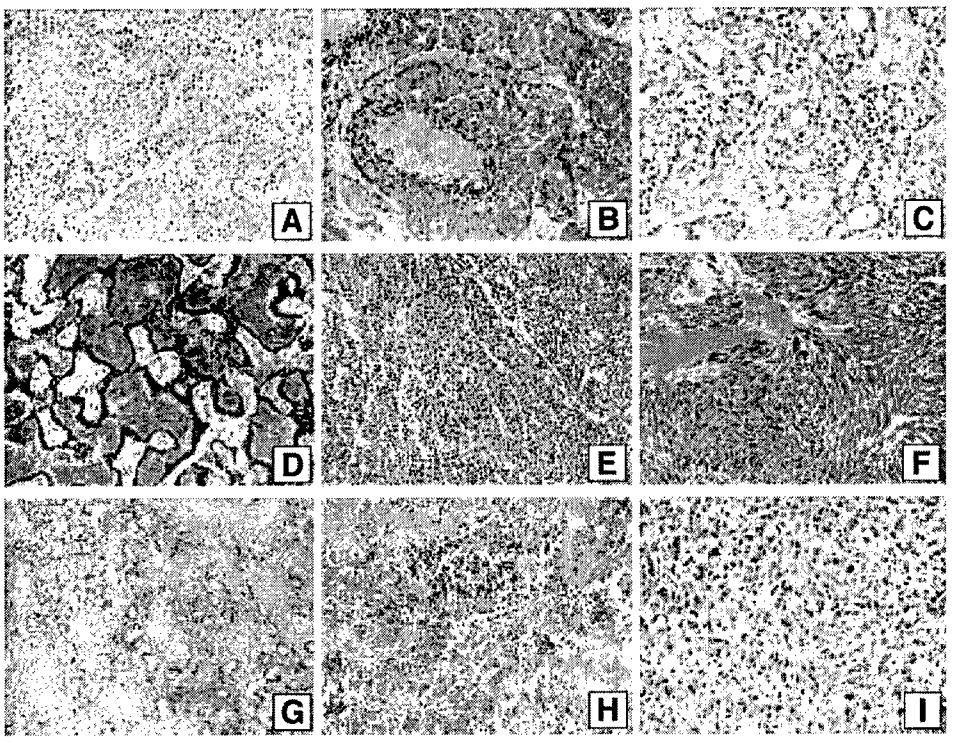

Fig. 2. *A–I*, hematoxylin and eosin stain. Histologic spectrum of treated metastatic prostate cancer: Solid sheets and nests of uniform tumor cells without (*A*, ×200), and with comedonecrosis (*B*, ×200) similar to Gleason grade 5, confluent cribriform glandular pattern of tumor similar to Gleason grade 4 (*C*, ×200), Neuroendocrine differentiation characterized by growth pattern of ribbons, trabaculae, and nests with uniform round nuclei, high N:C ratio, and salt and pepper chromatin (*D–E*, ×200), small-cell carcinoma, characterized by spindling and molding of the nuclei and high mitotic activity (*F*, ×400), uniform tumor cells with well-formed glands similar to Gleason grade 3 (*G*, ×200), tumor cells demonstrating poor cohesion with an undifferentiated growth pattern (*H*, ×400), and tumor with signet ring cell differentiation (*I*, ×200).

ation characterized by tumor growing in nests, trabaculae, and ribbons with uniform round nuclei, high N:C ratio, and salt and pepper chromatin (Fig. 2*D* and *E*). Only one of these three cases was seen as pure growth pattern, whereas in the remaining two cases, neuroendocrine differentiation was seen in combination with Gleason grade 5 pattern. Two cases had tumors with small-cell neuroendocrine carcinoma, similar to oat-cell carcinoma of the lung, characterized by spindling, crushing, and molding of the nuclei and high mitotic activity (Fig. 2*F*). Three cases had well-formed glands resembling Gleason grade 3 at some metastatic sites in addition to other growth patterns (three cases; Fig. 2*G*). In one case, metastatic tumors demonstrated poor cohesion with an undifferentiated growth pattern (Fig. 2*H*), and another case demonstrated focal signet ring cell differentiation (Fig. 2*I*) in addition to growth pattern of Gleason grade 5. The wide range of these varied histologic patterns is summarized and illustrated in Fig. 2 and Table 2. The distribution and overlap of different histologic patterns identified in these cases are schematically demonstrated in Fig. 1*B*. In one case, focal sarcomatoid differentiation was present only within the prostate site. Focal to diffuse nuclear pleomorphism within tumor cells was seen in 40% of cases, the remaining cases showed round-to-oval uniform nuclei often with prominent nucleoli, a bland cytology typical of prostate cancer.

Choice of Immunomarkers. Previous work by our group and others have described the immunophenotype of hormone-refractory metastatic prostate tumors (7, 8, 18–20). One limitation of our previous work has been the subjective manner in which protein expression was reported. Therefore, to attempt to provide more quantitative results and to study potential heterogeneity, we used the Chroma vision ACIS II system for the evaluation of several prostate cancer immunomarkers including PSA, AR, CGA, SYN, MIB-1, and AMACR. PSA was chosen because of its common use as a surrogate marker in the treatment of metastatic disease. AR was chosen because of its importance in understanding the development of androgen-independent disease. CGA and SYN were chosen to explore the concept of neuroendocrine differentiation in androgen-independent disease. MIB-1 was chosen as a proliferation marker. AMACR, a new molecular marker, has previously been shown to be a highly sensitive marker for clinically localized prostate cancer and colorectal cancer (3, 21). Our group recently demonstrated that AMACR expression is down-regulated in hormone-refractory metastatic prostate cancer, is androgen independent, and is likely related to tumor differentiation (22).

Immunophenotype of Prostate Cancer in Different Patients. The results are summarized in Table 2. The data present the median percentage staining with the range of the immunomarkers across all patients according to tissue site as present on the tissue microarray. All of the immunomarkers demonstrated considerable heterogeneity across disease sites. PSA expression was seen to vary for the percentage of PSA-positive cells with median expression of 39.3 (range, 0.3 to 99.44; SEM, 34.5; Table 2; Fig. 3). Consistent with its role as a transcription factor, AR was localized in nuclei. AR expression varied across tumor samples with 31% (83 of 265) of tumor samples expressing >50% AR and 41.5% (100 of 265) expressing <10% AR. Overall expression of AR was down-regulated with median AR expression of 20.04% (range, 0–100%, SEM, 34.28; Table 2; Fig. 3). The intensity of AMACR expression varied among the metastatic tumors; the median percentage of positive tumor cells was 8.18% (range, 0.19–71.97%; SEM, 14.58). The median MIB-1 expression was 4.55% (range, 0.25–26%; SEM, 4.61). CGA and SYN were infrequently observed in these metastatic prostate cancer cases, as we have reported previously (8). The median percentage of metastatic tumor cells demonstrating either CGA or SYN protein expression was 2.23% (range, 0.21–62.51; SEM, 7.55) and 0.83% (range, 0–49.11%; SEM, 8.21), respectively. Cases with neuroendocrine/small-cell morphology usually demonstrated neuroendocrine expression. We investigated whether any of the immunomarkers were predictive of survival time from the initiation of chemotherapy to death. Patients with a PSA median expression >50% demonstrated a significantly better survival than those with <50% expression ($P < 0.03$).

Immunophenotype of Prostate Cancer in the Same Patient. Having demonstrated the marked variation of immunomarkers in different organ sites and patients, we next investigated the expression of AR and PSA in tissue sites within the same patient. Tables 3 and 4 demonstrate the results for four different representative patients. Results are present as the median percentage staining. A range is given if there was more than one sample from that given site in a patient. All of the patients demonstrated marked differences in AR expression between different tissue sites (2- to 50-fold; Table 3). There was no pattern to the differences in AR expression between the different organ sites that could be distinguished, and this was true across all patients. Perhaps most striking was that several patients demonstrated a high amount of AR staining, although they were no longer responding to androgen-deprivation therapy. PSA expression also varied widely between tissue sites and, sometimes, even within the same tissue site of a patient (Table 4). For example, Case 30 demonstrated a range of PSA expression from a median of 3.03 in the prostate to a median of 60.61 in the bone, but the range of expression in the bone itself ranged from 14.67 to 77.86.

Expression Array Analysis of Different Metastatic Tissues. We next sought to determine whether the heterogeneity in histology and phenotype was mirrored by heterogeneity in gene expression. With expression array data that we have previously generated (6–8), we performed hierarchical clustering of the 16 metastatic samples from six cases in the rapid autopsy series. The metastatic adenocarcinoma samples were highly heterogeneous (Fig. 4). Only two metastases from a patient with small-cell histology demonstrated comparable cDNA expression results (Fig. 4). With the ONCOMINE database, we

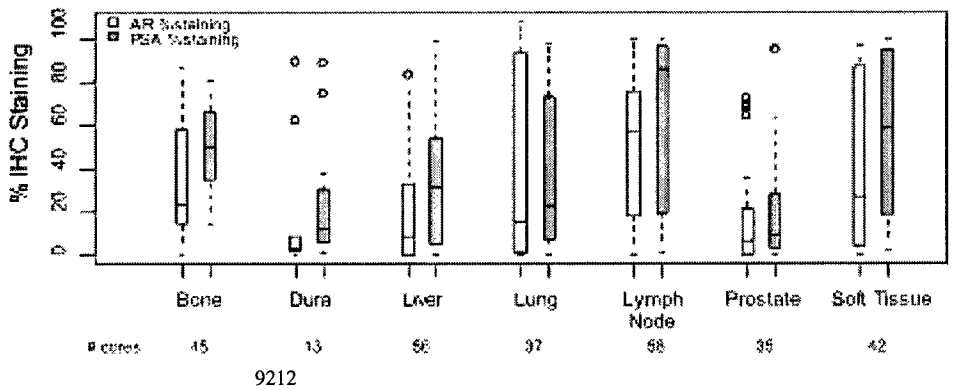

Fig. 3. Error bar graphs demonstrating variation of PSA and AR protein expression across the metastatic sites from 30 rapid autopsy cases. (*IHC*, immunohistography.)

Table 3 *Androgen receptor staining across metastatic sites for rapid autopsy (RA) cases 3, 14, 24, and 30*

Metastatic tissue	RA case 3	RA case 14	RA case 24	RA case 30
Adrenal	74.28 (64.4–78.93)			
Bone	22.66 (22.66–22.66)			81.07 (75.74–86.4)
Bladder				89.34 (88.54–96.65)
Dura	33.59 (5.17–62.01)		5.45 (1.86–9.04)	
Liver	46.22 (18.8–83.1)	0.48 (0.41–1.78)	2.28 (0.05–7.14)	
Lung	23.67 (15.41–27.4)	0.18 (0.1–0.22)	13.03 (5.64–41.95)	96.89 (85.49–99.56)
Lymph node	70.81 (40.78–94.65)		0.06 (0.06–0.06)	99.38 (99.01–99.58)
Meninges		0.73 (0.64–0.81)	0.01 (0–0.01)	68.93 (18.31–72.12)
Prostate	12.23 (3.7–13.61)	1.81 (1.29–2.33)		47.83 (16.3–87.04)
Soft tissue		19.99 (0.49–29.08)		
Testis	10.7 (10.7–10.7)			

NOTE. Median % staining of AR (range). There is one metastatic site less for cases 14 and 24 each of AR staining because of missing values. Tables 3 and 4 demonstrate heterogeneity in PSA and AR protein expression of metastatic sites in the same patient.

found that these tumors exhibited a similar expression pattern as small-cell lung cancer (23).

DISCUSSION

Autopsy series have been a valuable part of understanding the natural history of diseases, including prostate cancer (24–29). This study underscores the continued importance of the autopsy in modern medicine. It demonstrates that metastatic cancer is a group of diseases and needs to be treated as such and emphasizes the importance of obtaining metastatic tissue to understand the biology of prostate cancer. It is a widespread belief that prostate cancer metastasizes to osseous sites but rarely to visceral organs. Three groups (*i.e.*, University of Michigan, Ann Arbor, MI; University of Washington, Seattle, Washington; and Johns Hopkins University, Baltimore, Maryland) have developed rapid autopsy programs with the goal of procuring metastatic osseous and nonosseous prostate cancer samples for research purposes (2, 20). Our data and those of others demonstrate that prostate cancer commonly metastasizes to lymph nodes, liver, lung, adrenal, and dura sites in addition to the bone (20, 24). The MIB-1 data confirm the work of others in demonstrating the slow doubling time of prostate cancer, which suggests that the cancer develops in multiple sites over a long period of time (30, 31). These data also have implications for developing therapy because treatment based on using agents that act on cells with a rapid doubling time may not be effective in androgen-independent prostate cancer. The low rate of effectiveness of traditional chemotherapy agents in prostate cancer appears to reflect this finding.

We, as well as others, have identified several genes that are differentially expressed in primary *versus* metastatic disease (6, 7, 32). By definition, these analyses are designed to find potential similarities between samples at a similar disease stage and compare the two groups. Whereas these studies have identified several potential biomarkers, they were also remarkable for the amount of heterogeneity and overlap between primary and metastatic samples. To date, metastatic prostate tissue samples from the same patient and between patients have not been systematically been analyzed in an attempt to quantify this heterogeneity and to determine whether it may be important. Our data demonstrate that there are substantial differences in the genotype and phenotype of metastatic prostate cancer between patients and within the same patient. The data in Tables 2, 3, and 4, as well as in Fig. 3 demonstrate this heterogeneity when comparing metastatic sites. We did not investigate heterogeneity within individual metastatic sites by comparing multiple biopsies from a given site; however, it seems likely that heterogeneity would have been revealed at that level also. This will be the subject of future studies.

Normal prostate tissue and virtually all primary prostate cancer have been found to uniformly express AR; however, information of AR expression in hormone-refractory prostate cancer is limited (33). Our study demonstrates heterogeneity in AR expression with frequent AR-positive and AR-negative tumor populations between and within the same patient (Tables 2 and 3). In this study, overall AR expression is down-regulated in hormone-refractory prostate cancer, with 41.5% of tumor samples demonstrating $<10\%$ of AR, which suggests that, in such cases, an alternate AR bypass mechanism may also be important in the progression of androgen independence (34). However, the majority of patients still express substantial amounts of AR although they have undergone long-term androgen ablation. Because intracellular AR, a ligand-dependent transcription activator mediates androgen action, abnormalities in AR are believed to play an important role in the progression of prostate cancer (34–36). Chen *et al.* (37) have recently demonstrated that the AR in androgen-independent prostate cancer can still be active and fueled by submicromolar amount of testosterone. Our present study supports the observation that the AR still may play a central role in the biology of what has been traditionally termed "androgen-independent" disease.

Another clinically significant finding of this study is the low frequency of neuroendocrine phenotype in prostate cancer patients. It has been suggested that most androgen-independent prostate cancer has a neuroendocrine phenotype, and investigators have reported a correlation between the percentage of neuroendocrine expression with

Table 4 *Prostate-specific antigen staining across metastatic sites for rapid autopsy (RA) cases 3, 14, 24, and 30*

Metastatic tissue	RA case 3	RA case 14	RA case 24	RA case 30
Adrenal	98.54 (98.42–98.89)			
Bone	47.86 (44.63–51.08)	64.92 (57.03–72.82)	61.62 (61.62–61.62)	60.61 (14.67–77.86)
Bladder				2.84 (2.02–3.61)
Dura	47.27 (6.25–88.3)		2.27 (1.43–2.33)	5.84 (5.84–5.84)
Liver	39.41 (5.72–77.58)	96.03 (94.14–97.58)	20.14 (5.85–39.3)	
Lung	28.93 (7.87–49.09)	83.42 (12.43–97.61)	2.74 (1.47–95.45)	3.59 (0.34–14.07)
Lymph node	97 (86.18–98.29)		8.57 (8.57–8.57)	9.1 (8.53–13.27)
Meninges		41.65 (31.83–51.47)		
Prostate	61.67 (57.49–63.7)	4.27 (3.59–4.95)	13.05 (11.95–14.16)	3.03 (1.2–5.22)
Soft tissue		93.77 (83.49–98.61)		20.91 (3.91–39.04)
Testis	97.79 (97.79–97.79)			

NOTE. Median % staining of PSA (range). Tables 3 and 4 demonstrate heterogeneity in PSA and AR protein expression of metastatic sites in the same patient.

Fig. 4. Hierarchical clustering of 16 hormone-refractory metastatic prostate tumors from the rapid autopsy series demonstrates a small subset of genes that were specifically overexpressed in the small-cell phenotype (*lower left*) but not in the more typical glandular adenocarcinoma (see Discussion for details).

tumor progression and an adverse outcome (8, 20, 38, 39). Our data, as well as those of others, especially from the University of Washington, Seattle, suggest that this is not the case (20). In our series, only three patients demonstrated neuroendocrine phenotype by histology (two with pure small-cell histology and one with pure neuroendocrine differentiation but not reaching the threshold of small-cell carcinoma). Immunostaining for SYN and CGA was considerably variable and did not correlate with clinical outcome in the androgen-independent patients (as measured from the time of first chemotherapy).

We have extensively used these tumor samples from the rapid autopsy for research directed at understanding prostate cancer progression. Initial expression array analysis was critical in the identification of prostate- and cancer-specific genes such as *Hepsin*, *EZH2*, *MTA1*, and *TPD52*. (6, 7, 40) This initial work grouped all of the

hormone-refractory metastatic prostate cancer samples together for purposes of analysis and compared them with primary cancers as well as with normal tissue. These analyses were valuable in that they picked out dominant genes that were expressed differently over a majority of different tumor stages; however, these studies were not done by laser-capture microdissection and, therefore, did not take into account the heterogeneity of the tissues at similar stages. After review of the wide spectrum of histology in this study, we also questioned whether there was heterogeneity of gene expression when comparing metastatic tissue in androgen-independent disease. To our knowledge, this type of analysis, comparing metastatic samples against themselves, has not been done previously. We performed hierarchical clustering of 16 tumor samples from eight rapid autopsy cases. One case with two samples from two different sites had small-cell morphology. Whereas a few genes were differentially expressed in the majority of the metastatic sites including topoisomerase IIα, M_r 170,000 and procollagen-lysine, the majority of metastases did not share a similar gene expression pattern. These data demonstrate that the metastases share more differences than similarities in gene expression when compared with each other. These data were previously obscured when the metastases were used as part of larger arrays comparing normal and primary cancers with metastatic tissue. When we viewed the 95 most substantially differentially expressed genes, there were clusters of genes that were overexpressed only in the small-cell metastatic samples (Fig. 4). When small-cell prostate cancer does occur, it seems to have the genotypic pattern of small-cell lung cancer. With ONCOMINE, we were able to interrogate other over-60-expression array datasets that contained information on these differentially expressed genes (23). Multiple genes, including *TTF-1*, *PLOD2*, *TOP2A*, *Cyclin A2*, *CDC2*, *RBBP8*, and *GNAS*, found to be overexpressed in the two samples from a metastatic small-cell cancer of the prostate have all been previously identified as being significantly overexpressed in small-cell cancers of lung as compared with benign lung tissue as demonstrated by the adjusted *P*-values with ONCOMINE (40–43).

Previous studies have demonstrated the value of using PSA immunohistochemistry in the diagnosis of metastatic prostate cancer (44, 45). As previously noted by Stein et al. (45), the majority of end-stage prostate cancers retain PSA expression if all tumor samples are evaluated; however PSA expression is quite variable when individual tumor samples are evaluated (Tables 2, 4). There seemed to be no correlation between AR expression and PSA expression, which suggests that PSA expression may be driven by non-AR mechanisms in late-stage prostate cancer. We noted that patients with a median PSA staining of >50% had a longer survival in the androgen-independent setting than those with <50% staining. Roudier *et al.* (20) also found that that tumors with >50% of sites with >50% of cells expressing PSA were significantly associated with longer survival.

In conclusion, it is of note that no single biomarker or group of biomarkers has yet been identified that can successfully predict disease recurrence 100% of the time. Although studies have tried to identify subsets of genes that characterize the metastatic phenotype, these, by definition, ignore the heterogeneity of metastatic cancer (6, 7, 32). We demonstrate that end-stage hormone-refractory metastatic prostate cancer is a heterogeneous group of diseases. Understanding this heterogeneity is key to understanding prostate cancer progression and to guiding the development of future treatment paradigms.

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Dynamic Process of Prostate Cancer Metastasis to Bone

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Abstract Prostate cancer metastasis to the bone occurs at high frequency in patients with advanced disease, causing significant morbidity and mortality. Over a century ago, the "seed and soil" theory was proposed to explain organ-specific patterns of metastases. Today, this theory continues to be relevant as we continue to discover factors involved in the attraction and subsequent growth of prostate cancer cells to the bone. These include the accumulation of genetic changes within cancer cells, the preferential binding of cancer cells to bone marrow endothelial cells, and the release of cancer cell chemoattractants from bone elements. A key mediator throughout this metastatic process is the integrin family of proteins. Alterations in integrin expression and function promote dissociation of cancer cells from the primary tumor mass and migration into the blood stream. Once in circulation, integrins facilitate cancer cell survival through interactions between other cancer cells, platelets, and endothelial cells of the target bone. Furthermore, dynamic changes in integrins and in integrin-associated signal transduction aid in the extravasation of cancer cells into the bone and in expansion to a clinically relevant metastasis. Thus, we will review the critical roles of integrins in the process of prostate cancer bone metastasis, from the escape of cancer cells from the primary tumor, to their survival in the harsh "third microenvironment" of the circulation, and ultimately to their attachment and growth at distant bone sites. J. Cell. Biochem. 91: 706–717, 2004.

Key words: prostate cancer; integrins; metastasis; cell adhesion; bone; signal transduction; RhoGTPases

Prostate cancer is the second most common cause of cancer-related deaths among men in the United States [Jemal et al., 2003]. It is estimated that 220,900 new cases will be diagnosed and 28,900 deaths will occur in 2003. Approximately 90% of advanced stage prostate cancer patients develop bone lesions causing morbidity that includes bone pain, immobility, hematopoietic compromises, and spinal cord compression [Bubendorf et al., 2000; Rubin et al., 2000]. Current treatments are not curative, and patients have a median survival time of 9–12 months after becoming hormone refractory [Cheville et al., 2002].

Ellis et al. [2003] reported that prostate-specific antigen- (PSA-) expressing epithelial cells were detected in bone marrow samples from 60 of 126 (54%) patients with localized prostate cancer before radical prostatectomy, while only 33 of 138 (24%) patients had detectable PSA-expressing epithelial cells in peripheral blood. This finding supports the preferential enrichment of cancer cells in the bone marrow as an early metastatic event and leads to many interesting questions pertinent to prostate cancer metastasis. How do the cancer cells outgrow and escape from the primary site? How do they survive shear forces present in the circulation and evade immunosurveillance? How do these cells interact with the target bone endothelium? What factors in the bone microenvironment attract prostate cancer cells and prompt them to initiate growth? Understanding the biological processes leading to the establishment of clinically relevant bone metastases is not just an intellectual exercise as the answers to these questions may lead to invaluable therapeutic strategies to treat the currently incurable disease of advanced prostate cancer.

HISTORICAL PERSPECTIVE OF CANCER METASTASIS

Metastasis is a multi-step process that includes growth in a primary organ, neoangio-

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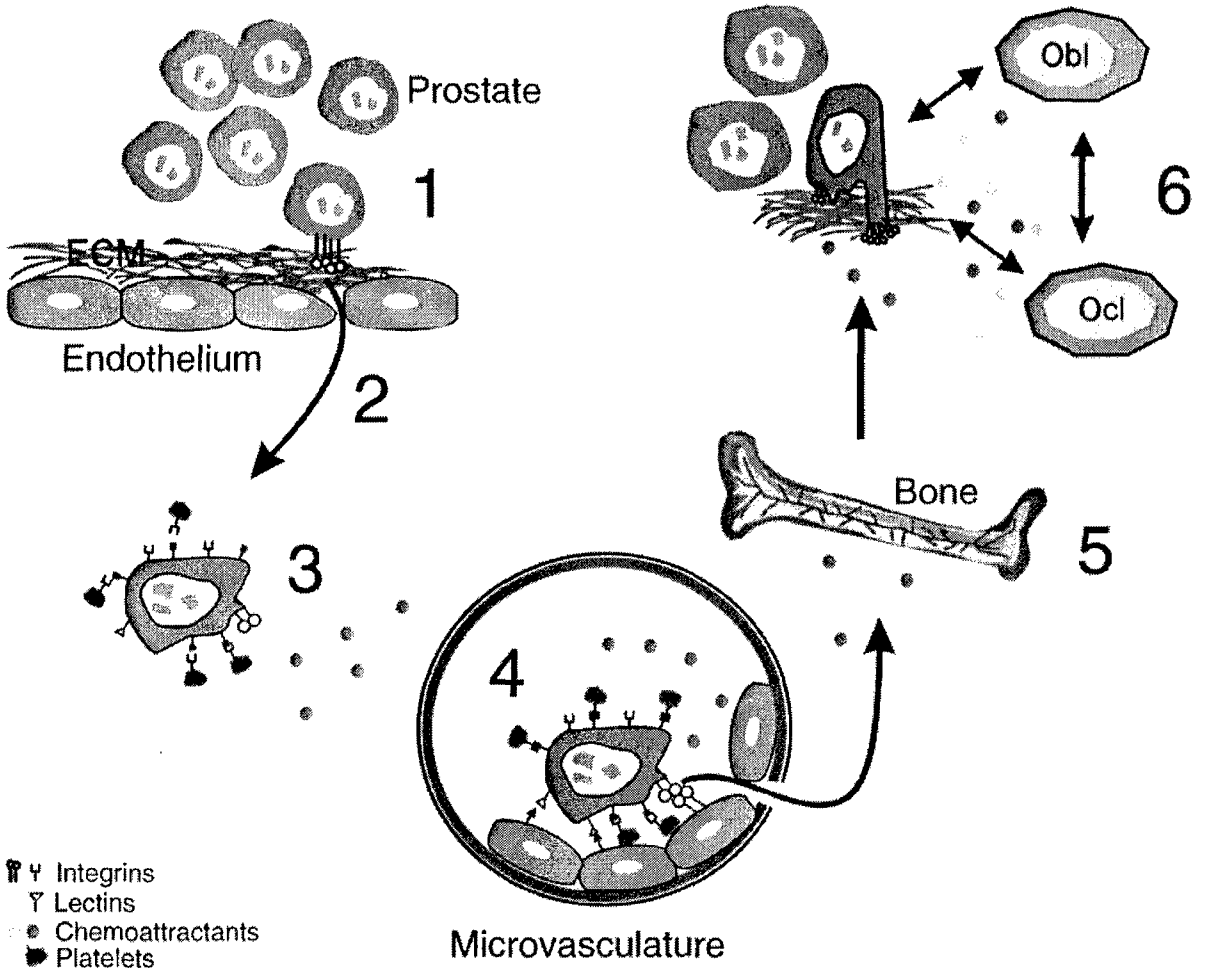

Fig. 1. Steps in prostate cancer metastasis to bone. Successful metastasis is a multi-step process that includes (1) growth and escape from a primary organ, (2) intravasation, (3) survival in circulation, (4) chemoattraction and extravasation, (5) and growth in bone through (6) cross-talks with osteoblasts and osteoclasts. These steps require the ability of cancer cells to adhere to and migrate across the surrounding extracellular matrix (ECM), actions mediated by the integrins.

genesis, intravasation into and survival in circulation, attachment to a distant target organ, extravasation at that site, and growth of a secondary neoplasm and, as such, appears to be an inefficient process (Fig. 1). While organ-specific localization of luciferase-labeled PC-3 human prostate cancer cell line cells was visualized by non-invasive imaging 15 min post-intracardiac injection in immunocompromised mice, no viable cells were detected 24 h later. Despite this indication that most of the injected cells were either dead or metabolically inactive, skeletal and soft tissue micrometastases were apparent on imaging several days later and were confirmed histologically and radiographically weeks later [Rosol et al., 2003]. Similarly, intravital videomicroscopy of various types of cancer showed that only 2% of cancer cells formed micrometastases [Luzzi et al., 1998; Varghese et al., 2002]. Furthermore, 99% of these micrometastases failed to form larger tumors, although numerous solitary cells remained detectable in the tissue months after injection [Naumov et al., 2001, 2002; MacDonald et al., 2002].

Stephen Paget [1889] was the first to present an explanation for the non-random patterns of cancer metastases. His "seed and soil" theory proposed that there was something about metastatic sites that promoted cancer cell growth similar to the tendency of seeds to grow in fertile soil, i.e., that factors in the environment at a metastatic site contributed to the proliferation of cancer cells there. Forty years later, James Ewing [1928] presented a different view

proposing that cancer cells grew at a particular site because they were directed to that site by the direction of blood flow and lymphatics. It is likely that both of these theories are correct, at least in part. Ewing's theory accounts for cancer growth in the draining lymph nodes and the liver, but Paget's theory describes distal metastases that are organ-specific, such as metastases to the bone.

Isaiah Fidler [2002] redefined the modern "seed and soil" hypothesis as three principles. First, cancerous tissues contained heterogeneous subpopulations of cells with different angiogenic, invasive, and metastatic properties. Second, the metastatic process was selective for cells that survived the long journey to a distal organ. Finally, the success of the metastatic cells depended on the ability of those cells to interact and to utilize the "soil" provided in their new microenvironment. These properties are demonstrated by co-culture of an isolated mouse femur with PC-3 human prostate cancer cells (Fig. 2). As shown by scanning electron micrograph, after 3 days cells exhibiting diverse phenotypes are attached to the bone. We hypothesize that these variable morphologies may represent the heterogeneous population of prostate cancer cells that differentially responds to growth on bone. Alternatively, these pleomorphic cells may be in different stages of metastasis, as some appear anchored to the bone at the extended edges, perhaps "feeling" out the environment for chemotactic factors, while others seem to have formed firm adhesions with the bone, suggesting that they may have begun an invasive process.

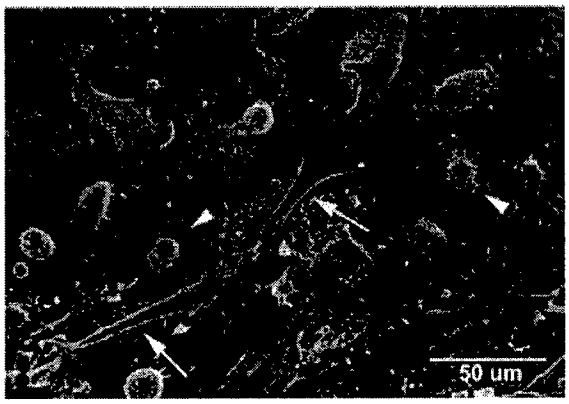

Fig. 2. Adhesion and invasion of PC-3 cells into mouse bone in an explant model. Several different cell morphologies are evident when PC-3 cells were co-cultured on mouse bone explant for 3 days, fixed with 2.5% glutaraldehyde in Sorenson's buffer and prepared for scanning electron microscopy. These cells possibly represent either a heterogeneous population of prostate cancer cells or cells in different stages of bone metastasis. Some cells are elongated on the bone, perhaps in search of chemotactic factors (arrows). Other cells exhibit halo-like area around themselves, suggesting that they have initiated the invasion process (arrowheads).

METASTATIC PROCESS AND INTEGRINS

In normal prostate development, the interaction of prostate epithelial cells with surrounding stroma influences their growth, survival, and differentiation potential. Components of the surrounding stroma include numerous cell types, such as fibroblast, endothelial, neuroendocrine, and inflammatory cells; soluble growth factors; and insoluble laminin-rich extracellular matrix (ECM). Many of the steps in cancer metastasis involve changes in cell adhesion to adjacent cells and to the ECM. The cell surface receptor integrins have been implicated in these events since they mediate homotypic and heterotypic interactions of prostate cancer cells within their microenvironment. Integrins are composed of non-covalently associated α and β subunits that play a role in mediating both cell–cell interaction and cell–matrix interaction (Fig. 3). To date, genes for 16 α and 8 β subunits have been identified. Both types of subunits encode single-pass transmembrane proteins with short cytoplasmic tails, except for the β4 subunit which contains more than 100 residues [Longhurst and Jennings, 1998; Mizejewski,

Fig. 3. Overview of the integrin signaling pathway at the focal adhesion. Modulation of the integrins through RhoGTPases leads to changes in cell motility (see text for details). Activation of the G-protein protease-activated receptor 1 (PAR1) by thrombin receptor-activating pepetide (TRAP) is shown as an example in more motile (fibroblastoid) cells, Cdc42 regulates filopodia formation to allow direction sensing of chemotaxis upon stimulation. Once the direction has been established, Rac activity leads to lamellipodium extension and new focal adhesions in the direction of the gradient. Increased activation of Rac and Cdc42 leads to the down-regulation of the Rho signaling pathway that regulates stress fiber formation, but activates contractile ring formation, leading to cell migration. In less motile (epitheloid) cells, cross-talks among the RhoGTPases lead to stress fiber formation in the cell, thereby inducing cell spreading and formation of high affinity integrin binding. **Panel A:** Represents a scanning electron micrgraph of untreated control PC-3 cells cultured on Thermanox coverslips. **Panel B:** Represents PC-3 cells 1 h after treatment with 10 μM TRAP to activate the thrombin receptor, which leads to membrane ruffling and filopodium extensions (Adapted from [Evers et al., 2000]).

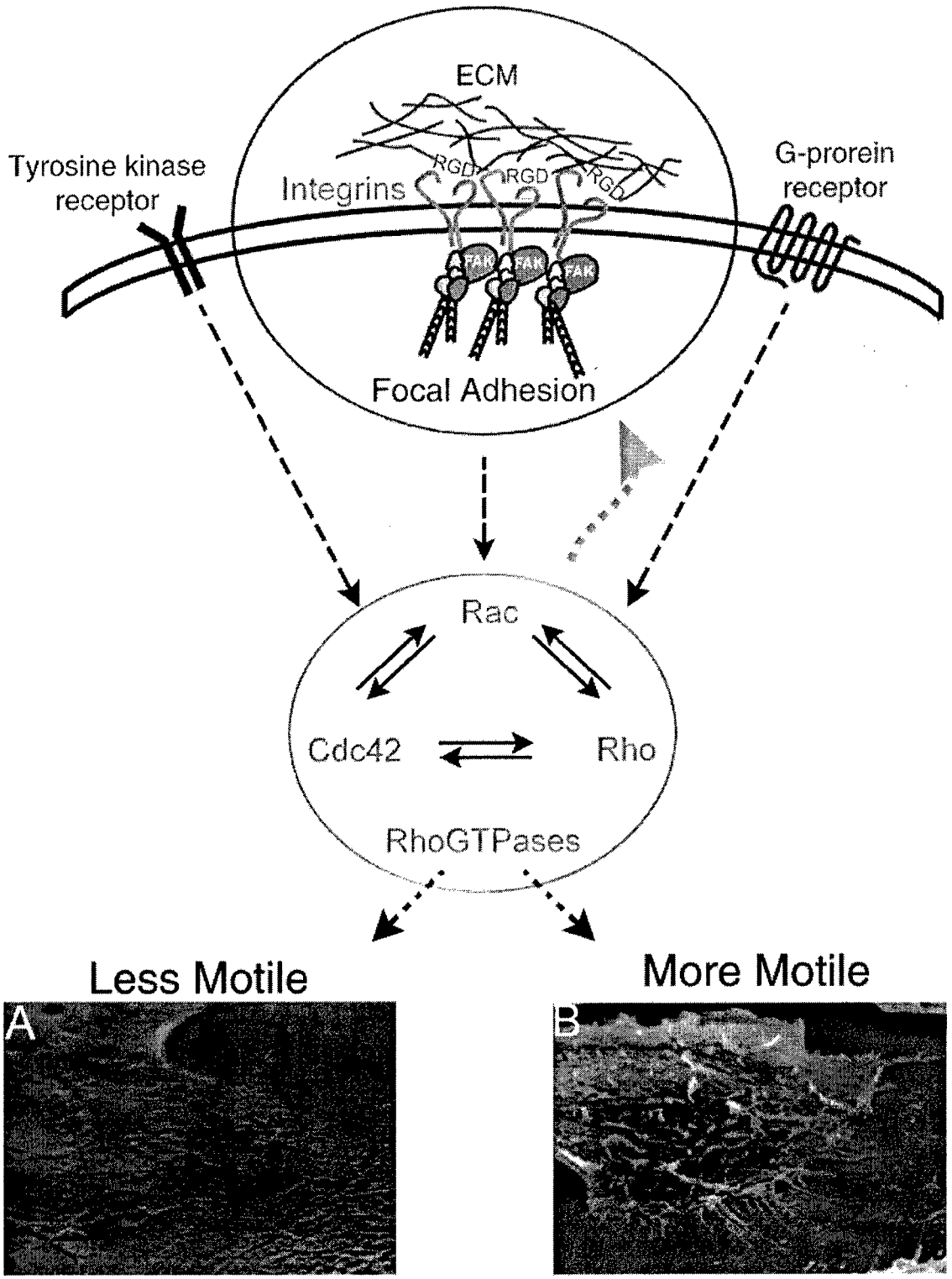
ECM
Tyrosine kinase receptor
Integrins
RGD
RGD
RGD
G-prorein receptor
FAK
FAK
FAK
Focal Adhesion
Rac
Cdc42
Rho
RhoGTPases
Less Motile
More Motile
A
B

Fig. 3.

TABLE I. Involvement of Integrins in Prostate Cancer Metastasis

	Integrins	Ligand*	Role in prostate cancer metastasis	Reference
Primary tumor	α6β4	Laminin	Acinar morphogenesis	[Mercurio et al., 2001;
	α6β1	Laminin	Migration, invasion	Bello-DeOcampo et al., 2001b]
Circulatory system	αIIbβIIIa	Fibrinogen, platelets	Microembolism, arrest in circulation	[Trikha et al., 1998]
	αvβ3	Vitronectin, endothelial cells	Arrest in circulation	[Romanov and Goligorsky, 1999; Pilch et al., 2002]
Bone	α2β1	Collagen	Growth stimulation	[Zheng et al., 2000; Kiefer
	α3β1	Collagen	Migration, invasion	and Farach-Carson, 2001]
	αvβ3	Osteopontin, osteonectin	Growth stimulation, migration, invasion	[Festuccia et al., 1999a; Angelucci et al., 2002]

*The major contributory role of integrin-mediated cell adhesion in prostate cancer metastasis is summarized. Although each integrin receptor can bind to multiple ligands and some ligand has multiple integrin receptors, only the contents of the ECM that are important in prostate cancer progression are listed.

1999]. The α subunits contain Ca^{2+}-binding sites on the extracellular domain linked by a disulfide bond to the transmembrane portion [Argraves et al., 1987]. The β subunits contain four repeating units of cysteine-rich motifs proximal to the transmembrane region which are joined together by disulfide bonds [Argraves et al., 1987]. At least 22 different α/β heterodimers are known, and it is the particular combination of α and β subunits that determines ligand binding specificity. For example, fibronectin uniquely binds to the integrin heterodimer of α5 and β1 [Argraves et al., 1987].

ESCAPE FROM THE PRIMARY TUMOR SITE

Changes in integrin expression have been documented in primary prostate tumors and prostate cancer cell lines compared to normal prostate tissue (see Table I). Immunohistochemical studies of normal, prostatic intraepithelial neoplasia (PIN), and cancerous prostate tissues indicate that loss of the laminin receptor α6β4 integrins occurs with increasing malignancy [Davis et al., 2001]. Low Gleason sum score correlates with increased expression of the α3 and α6 integrin subunits, while high Gleason sum score correlates with low expression of α3 and negative expression of α6 integrin subunits compared to normal prostate tissue [Schmelz et al., 2002]. Furthermore, β1c integrin, an alternatively spliced variant of the β1 subunit abundantly expressed in normal prostate gland, inhibits cell proliferation [Fornaro et al., 1998] and is down-regulated in prostate carcinoma [Fornaro et al., 1996]. These changes in integrin expression are modulated upstream by many different factors including hormones and growth factors. For example, androgen-independent PC-3 prostate cancer cells transfected with androgen receptor (AR) express lower cell surface α6β4 integrins than the parental cells [Bonaccorsi et al., 2000]. Furthermore, these AR-positive cells exhibit decreased invasion through Matrigel, a laminin-rich reconstituted basement membrane, and less adhesion to laminin, thereby correlating reduced expression of α6β4 integrins with decreased invasiveness. Similarly, over expression of parathyroid hormone-related protein in PC-3 cells increases cell surface integrin expression and leads to an increase in cell adhesion to ECM proteins [Shen and Falzon, 2003].

The influence of integrin cell surface expression and interaction with the ECM in early prostate cancer progression is also illustrated by prostate acinar morphogenesis using a series of human prostatic epithelial cell lines. Nontumorigenic RWPE-1 prostate epithelial cell line form acini when grown in three-dimensional Matrigel cell cultures, while invasive WPE1-NB26 cells fail to form acini. In addition, RWPE-1 cells form acini on laminin-1, but not collagen or fibronectin, and are unable to form these structures when exposed to blocking antibodies for laminin-1 and laminin integrin receptor α6 or β1 subunits [Bello-DeOcampo et al., 2001a]. WPE1-NB26 cells show a lack of α6 integrin expression as well as abnormal β1 integrin expression [Bello-DeOcampo et al., 2001b]. Thus, alterations in the expression of laminin integrin receptors correlate with the ability of prostate cancer cells to escape the laminin-rich ECM support at a primary tumor site. This is further illustrated by the ability to accelerate rates of tumor growth in immunocompromised mice injected with prostate cancer cells suspended in Matrigel, which is abrogated

by the inclusion of laminin cell adhesion peptide YIGSR [Passaniti et al., 1992]. Secretion of proteolytic enzymes by prostate cancer cells permits the cells to digest through the basement membrane to reach the microvasculature. Laminin-5 is a crucial protein in mediating cell stability, migration, and anchoring filament formation. Invasive prostate carcinoma shows a lack of protein expression of β3 and γ2 chains of laminin-5 and altered expression of the α3 chain [Hao et al., 2001]. Cleavage of laminin-5 by membrane-type 1 matrix metalloproteinase enhances migration of DU145 prostate carcinoma cells by two-fold compared to migration on uncleaved Laminin-5 thus promoting the escape of prostate cancer cells from the primary site [Udayakumar et al., 2003].

SURVIVAL IN THE CIRCULATORY SYSTEM

After prostate cancer cells cross the surrounding stroma, they enter the microvasculature in a process called intravasation and must evade assault by the immune system and the simple shear forces of blood flow. Cells appear to travel through the blood as part of a fibrin clot surrounded by other cancer cells and platelets to survive [Walz and Fenton, 1994], with platelets binding to cancer cells via the integrins αIIbβIIIa. Treatment with either anti-platelet antibodies or heparin inhibits platelet-tumor cell interaction [Borsig et al., 2001] and reduces lung metastasis [Stoelcker et al., 1996]. Similarly, blocking antibodies to αIIbβIIIa inhibits lung colonization of mouse tail vein-injected DU145 cells [Trikha et al., 1998].

In the vasculature, thrombin may mediate cancer cell–platelet adhesion [Cooper et al., 2003] and may activate resting platelet integrins αIIbβIIIa to induce platelet aggregation [Trikha and Nakada, 2002]. As well, activation of protease-activated receptor 1 (PAR1) on cancer cells by thrombin increases cell adhesion to platelets [Walz and Fenton, 1994]. Furthermore, we have previously shown that VCaP and PC-3 prostate cancer cell lines, both derived from osseous metastases, have increased PAR1 expression compared to normal prostate tissue [Chay et al., 2002]. Thus the ability of cancer cells to adhere and aggregate with platelets through the expression of cell surface receptors may protect them from shear stresses of the circulation and facilitate arrest in the microvasculature.

INTERACTION WITH THE ENDOTHELIUM

Hemodynamics may bring cancer cells into the bone marrow, but it alone does not explain the high frequency of prostate cancer metastasis to the bone [Yoneda, 1998]. Perhaps the presence of chemotactic gradients in the bone sinusoids contributes to the attraction, but the role cell–cell interaction plays cannot be ignored. We have shown that prostate cancer cells preferentially bind to bone marrow endothelial cells three- to five-fold more than to aortic, umbilical vein, or dermal vascular endothelial cells [Lehr and Pienta, 1998; Cooper et al., 2000b]. Furthermore, prostate cancer cells adhere preferentially directly to these bone endothelial cells and not to ECM proteins present in the bone in vitro, although the growth of bone marrow endothelial cells on bone ECM components significantly increases their affinity for PC-3 cells [Cooper et al., 2000b].

Adhesion and extravasation of prostate cancer cells from fenestrated bone marrow endothelium most likely occurs as a complex set of interactions between bone marrow endothelial cells, bone ECM components, and bone marrow stromal cells. The "dock and lock" mechanism was proposed as one explanation for extravasation and is similar to the inflammatory response of leukocytes [Honn and Tang, 1992] as both processes involve the arrest of circulating cells on the endothelium by low-affinity binding, induction of a firmer cell adhesion, extravasation, and invasion of the surrounding matrix [Buck, 1995]. During the "docking" step of inflammatory response, induced expression of P-selectin, a type of cell adhesion molecule on platelets, leukocytes, and endothelial cells, on activated endothelial cells is responsible for the low affinity binding of leukocytes to endothelial cells [Meyer and Hart, 1998]. Similarly, interaction of P-selectin with its ligand sialyl Lewis^x carbohydrates is believed to cause the arrest of cancer cells in complex with platelets and leukocytes [Chopra et al., 1990; Bhatti et al., 1996; Borsig et al., 2001]. Indeed, elevated expression of sialyl Lewis^x is detected at the surface of cancer cells and correlates with poor prognosis in prostate cancer [Martensson et al., 1995], and antibodies against sialyl Lewis^x block adhesion of neutrophils and tumor cells to endothelial cells and platelets [Geng et al., 1990]. Furthermore, fewer lung metastasis and slower tumor growth occur when colon cancer

cells are implanted into P-selectin-deficient mice [Kim et al., 1998]. In breast and prostate cancer cells, adhesion to the microvascular endothelium of metastasis-prone tissues is also mediated in part by interactions between cancer-associated Thomsen–Friedenreich (TF) glycoantigen (Galβ1-3GalNAc) presenting on neoplastic cells and β-galactoside binding lectin galectin-3 expressing on endothelium [Lehr and Pienta, 1998; Ellerhorst et al., 1999; Glinsky et al., 2000, 2001; Nangia-Makker et al., 2002; Khaldoyanidi et al., 2003]. This adhesion is abrogated by blocking antibodies to β-galactoside-binding lectin, galectin-3, or TF antigen [Lehr and Pienta, 1998; Glinsky et al., 2003].

Similar to inflammatory response of leukocytes, the "locking" of prostate cancer cells to endothelial cells is facilitated through the complex collaboration of integrins. We have previously reported that β1 integrin was not involved in PC-3 prostate cancer cell–endothelial cell adhesion [Cooper et al., 2000a]. However, a more recent study showed that blocking antibodies to this subunit inhibited adhesion of PC-3 cells to bone marrow endothelial cells by 64% [Scott et al., 2001]. These conflicting results suggest the involvement of other integrins or cell adhesion molecules in prostate cancer cell adhesion to the endothelium. Indeed, cooperativity between αvβ3, α5β1 and α3β1 integrins is necessary for PC-3 and DU145 cell adhesion to interleukin-1-stimulated human umbilical vein endothelial cells [Romanov and Goligorsky, 1999].

BONE MICROENVIRONMENT

Once cancer cells have reached the bone, they must utilize the bone microenvironment to survive and propagate. In a review of bone scans from 27 patients with limited skeletal involvement, the distribution pattern of early prostate cancer metastases was similar to the distribution of normal adult bone marrow [Imbriaco et al., 1998]. This observation supports Ewing's theory that cancer cell delivery to the bone is simply a reflection of the volume of blood flow [Ewing, 1928]. However, theories of preferential adhesion and the potential role of chemoattractants in colonization and subsequent growth are not discounted [Keller et al., 2001; Taichman et al., 2002; Cooper et al., 2003] as there is little doubt that the bone microenvironment provides a rich "soil" for the prostate cancer cell "seeds" [Paget, 1889]. For example, bone extracts induce at least a three-fold increase in invasion by PC-3 and DU145 cells compared with brain and other tissue extracts, demonstrating that bone contains significant migration and chemoinvasion promoting factors for prostate cancer cells [Jacob et al., 1999]. By purifying the bone extract, osteonectin was identified as the chemoattractant that promoted prostate cancer cell invasion [Jacob et al., 1999]. Blocking antibodies to the αvβ3 integrins have been shown to reduce prostate cancer cell adhesion to crude bone protein extract by 94% [Hullinger et al., 1998], suggesting the importance of the integrins in the process.

Numerous other factors contribute to prostate cancer cell proliferation in the bone, and many are mediated through the engagement of integrin receptors. Unlike prostate epithelial associated ECM, the main component of bone ECM is collagen type I which is a ligand for α2β1 and α3β1 integrins. Greater proliferation rates for prostate cancer cells are observed in cells grown on collagen I compared to plastic or fibronectin substrates; cell signaling through phosphatidylinositol 3-kinase (PI3K) and increased expression of cyclin D1 are implicated in this process [Kiefer and Farach-Carson, 2001]. Interestingly, osteopontin, a non-collagenous bone ECM component, stimulates proliferation of quiescent prostate epithelial cells more than collagen in an integrin-mediated manner [Elgavish et al., 1998], stimulates anchorage-independent growth of the human prostate cancer cell lines LNCaP and C4-2 [Thalmann et al., 1999], and induces PC-3 cell migration and invasion via αvβ3 integrin function [Angelucci et al., 2002].

Although more than 95% of the bone ECM is composed of collagen type I, other proteins are also deposited by osteoblasts during bone formation [Hauschka et al., 1986]. Co-culture of PC-3 cells with osteoblasts reveals that transforming growth factor-β1 (TGF-β) produced by osteoblasts stimulates PC-3 cell migration and invasion as well as increases α2β1 and α3β1 integrins expressions [Festuccia et al., 1999a]. Furthermore, osteoblast-conditioned medium stimulates the release of proteolytic enzymes urokinase plasminogen activator and matrix metalloproteinase-9 from prostate cancer cells [Festuccia et al., 1999b].

Although factors in the bone microenvironment promote prostate cancer cell growth,

prostate cancer cells also contribute to bone remodeling in a "vicious cycle" [Chung, 2003]. Because prostate cancer metastases are usually osteoblastic in nature, the role of bone morphogenetic proteins (BMPs) in the course of bone metastasis is quite intriguing since they contribute to bone formation. Bentley et al. [1992] first reported that the expression of BMP-6, a member of the TGF-β superfamily, was detected in prostate tissue samples of over 50% of patients with clinically defined metastatic prostate cancer, but not non-metastatic or benign prostate samples. Subsequent studies have confirmed the increased expression of BMP-6 in metastatic prostate cancer cells [Barnes et al., 1995; Hamdy et al., 1997; Autzen et al., 1998; Thomas and Hamdy, 2000]. It is believed that secretion of BMP-6, among other proteins, by prostate cancer cells contributes to osteoblastic lesions because BMP-6 stimulates osteoblastic differentiation of pluripotent mesenchymal [Ebisawa et al., 1999]. Furthermore, osteoblastic differentiation requires the activation of focal adhesion kinase (FAK), an immediate effector of the integrin signaling pathway [Tamura et al., 2001].

INTEGRIN REGULATION OF MOTILITY AND DYNAMIC CELL–SUBSTRATE INTERACTIONS

As discussed above, many steps of the metastatic cascade involve the establishment and termination of adhesive interactions between cancer cells and the ECM via integrins. However, integrins also regulate intracellular signaling pathways that control cytoskeletal organization, force generation, and survival [Hood and Cheresh, 2002]. This signal transduction is modulated in two directions: activation of integrins to bind to ligands produces "inside-out" signaling and generation of ligand bound integrins activates downstream intracellular kinases and GTPases causing "outside-in" signaling. In this "outside-in" signaling, ligand bound integrins cluster with structural and catalytic focal adhesion-associated proteins at cell–ECM junctions called focal adhesions. Because integrins do not possess kinase activity, the focal adhesion-associated protein FAK initiates the intracellular signaling cascade using its ability to recruit downstream effectors to the focal adhesion [Hood and Cheresh, 2002]. In prostate cancer, the highly tumorigenic PC-3 and DU145 cell lines have increased expression of FAK compared to the poorly tumorigenic LNCaP cells, suggesting a differential modulation of the integrin signaling pathway in metastatic prostate cancer cells [Slack et al., 2001].

Cell motility are a coordination of focal adhesion assembly at the leading edge of motile cells, providing traction for cell migration, and focal adhesion disassembly at the trailing edge of these cells, resulting in forward movement [Sastry and Burridge, 2000]. RhoGTPases, activated through integrin-mediated, focal adhesion-localized FAK and other tyrosine kinases, and G-protein receptors, appear to be key facilitators of these dynamics. These proteins and their associated downstream signals are elevated in malignant tissues but are almost non-existent in normal tissues and benign hyperplasias [Fritz et al., 2002; Kamai et al., 2002; Kostenuik et al., 1996].

Currently there are at least 18 known members of RhoGTPases. The best-characterized are Cdc42, Rac, and Rho [Sahai and Marshall, 2002]. Activated Cdc42 produces filopodia, actin-rich spikes that establish cell polarity by sensing tactic signals [Arthur and Burridge, 2001]. These extended antennae allow cells to detect changes in their surrounding and transduce intracellular signals to adjust to their microenvironment, a property which is crucial in cancer metastasis. Activated Rac coordinates focal adhesion assembly in lamellipodium and membrane ruffling [Ridley et al., 1992; Nobes and Hall, 1995; Clark et al., 1998]. These new focal adhesions establish a path for cancer cells to begin their quest to find more enriched "soil." Activated Rho generates contractile forces that push a cell body toward the leading edge and can stimulate other downstream signaling pathways leading to stress fiber formation, cell contraction, or actin polymerization depending on cross-talk between Rac, Cdc42, and other Rho regulatory proteins [Ridley, 2001]. For example, inhibition of Rho kinase, a Rho downstream target, decreases prostate cancer cell chemotactic migration in vitro and tumor growth and angiogenesis in vivo [Somlyo et al., 2000, 2003]. As well, a putative Rho regulatory protein was recently identified that encodes a novel Src homology 3 domain-containing guanine nucleotide exchange factor (CSGEF) [Qi et al., 2003]. Expressed only in prostate and liver tissues, CSGEF mRNA levels are increased two-fold in the LNCaP prostate cancer

cell line after androgen treatment, suggesting a possible role of CSGEF in modulating prostate cancer cell metastasis. G-protein receptor PAR1 activation of Rho similarly induces actin cytoskeletal reorganization (reviewed in [Whitehead et al., 2001]). In prostate cancer cells, this activation contributes to increased prostate cancer invasiveness [Chay et al., 2002; Cooper et al., 2003; Greenberg et al., 2003] and increased production of stress fibers in LNCaP cells treated with thrombin [Greenberg et al., 2003].

PROSPECT: CAN INTEGRINS BE USED AS TARGETS TO PREVENT OR TREAT BONE METASTASES?

Prostate cancer cells with varying degrees of malignancy exhibit differential expression of integrin receptors which leads to the ability of these cells to develop preferential binding or "sensing" of the microenvironment through different integrins [Edlund et al., 2001]. This raises questions to the importance of integrins in prostate cancer bone metastasis and to their feasibility as therapeutic targets. Unfortunately, the exact integrins involved in the various steps of prostate cancer metastasis remain undefined (See Table I). Furthermore, the signaling pathways responsible for the regulation of the cell surface expression of integrins are unclear. For example, will inhibiting the "sensing" mechanism of the cancer cells through inhibition of the Cdc42 signaling pathway be sufficient to suppress metastasis? Or is there other mechanism(s) at work? The affinity and avidity of the integrin receptors can be modulated by many factors both from the outside and the inside of cells. Weakly attached cells cannot generate enough force for movement whereas highly adhesive forces can render cell immobility. We know that the ECM protein substrates regulate the affinity of the integrins, but what factors are regulating the avidity of the integrins? Zheng et al. [2000] have shown that LNCaP cells use the $\alpha v\beta 3$ integrins to adhere to vitronectin and osteopontin. However, $\alpha v\beta 3$ mediated cell migration and PI3K activation, through its downstream serine/threonine kinase Akt, upon interaction with vitronectin, whereas adhesion to osteopontin did not induce $\alpha v\beta 3$-mediated cell migration and PI3K/AKT pathway activation, thus suggesting some unknown mechanism co-modulating the "outside-in" signaling pathway. Growth factors and signaling through the G-protein-coupled receptors have been known to activate RhoGTPases (reviewed in [Kjoller and Hall, 1999]). Which factor(s) in the microenvironments of the primary tumor, the circulation, and the target organ can activate integrin-mediated adhesion, dynamic cell structure, migration, and invasion? The answers to these questions will lead not only to a better understanding of the biology of prostate cancer metastasis but should also lead to the identification of therapeutic targets for the prevention ant treatment of prostate cancer metastasis.

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